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Regulation of rod photoreceptor phosphodiesterase (PDE6) by the glutamic acid -rich protein 2 (GARP2)

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REGULATION OF ROD PHOTORECEPTOR PHOSPHODIESTERASE (PDE6) BY THE GLUTAMIC ACID-RICH PROTEIN 2 (GARP2)

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ABSTRACT

REGULATION OF ROD PHOTORECEPTOR PHOSPHODIESTERASE (PDE6) BY THE GLUTAMIC ACID-RICH PROTEIN 2 (GARP2)

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Rod photoreceptor phosphodiesterase (PDE6), the central enzyme of visual transduction in vertebrate photoreceptors, associates with the disk membranes of the rod outer segment (ROS) of the photoreceptor cell. This association insures the high efficiency of activation by the G-protein, transducin, and the precise control of its inactivation. In addition to binding to transducin during visual excitation, PDE6 is hypothesized to be regulated by other interacting proteins.

The first aim of this research was to isolate and identify the proteins that interact with PDE6 during various stages of the visual signaling pathway. We evaluated methods for solubilizing PDE6 and its binding partners from the ROS disk membrane, and analyzed the protein composition by immunoprecipitation or size exclusion chromatography. Our results suggested that additional, as-yet unidentified, proteins bind to PDE6 in its nonactivated and activated states. This work led to enhanced protocols for purifying rod and cone PDE6 from photoreceptor cells.

The second aim of the study was to evaluate the importance of the Glutamic Acid-Rich Protein-2 (GARP2) as a PDE6-interacting protein. First, we confirmed earlier observations that GARP2 is a high-affinity PDE6 interacting protein, and is present in amounts sufficient to stoichiometrically bind PDE6. Addition of purified GARP2 to ROS membranes containing PDE6 demonstrated that it is able to inhibit the basal activity of the nonactivated PDE6 holoenzyme. In contrast with a previous report, GARP2 does not alter the ability of transducin to activate PDE6. These results suggest a role for GARP2 in reducing the spontaneous activation of dark-adapted PDE6, thereby serving to increase the light sensitivity of rod photoreceptors as "single photon detectors."

The final aim was to investigate the molecular mechanism by which GARP2 interacts with PDE6 and regulates its catalytic properties. We found that GARP2 exerted a destabilizing effect on the regulatory cGMP binding sites of the PDE6 catalytic dimer, as well as interacting directly with two distinct subdomains of the inhibitory subunit of PDE6. These latter interactions are believed to account for the observed ability of GARP2 to enhance the affinity of the inhibitory subunits for the catalytic dimer of PDE6.

CHAPTER 1

INTRODUCTION[1]

1. Photoreceptor Cells and Visual Transduction in Vertebrates.

In vertebrates, vision starts in specialized neuronal cells located in the retina: rods and cones. Rods are significantly larger in the vertebrate retina, and more abundant than cones (see. Fig1.1.). In mammalian retina, cones are grouped in a central region named

Figure 1.1. Electron microscopy visualization of rods and cones

[1] The abbreviations used are: PDE6, photoreceptor phosphodiesterase; Pγ, inhibitory 10 kDa γ subunit of PDE6; GARP2, glutamic acid-rich protein 2; PrBP-δ, 17 kDa prenyl-binding protein; ROS, rod outer segment; Tα, G-protein, transducin, α subunit.

fovea. The internal organization of photoreceptor cells consists of two major compartments: the inner segment and the outer segment (Fig. 1.2.). The two subcellular compartments are connected through a ciliary region. The inner segment contains all the cellular machinery of a typical cell: the nucleus, mitochondria and other organelles. At the end of the inner segment is the synaptic terminus which communicates the light response to other retinal neurons.

The outer segment is the highly specialized part of the photoreceptor, having a unique morphology and containing all the proteins involved in phototransduction. Rods and cones outer segments have different wavelength specificity for light reception. Rods are responsible for night vision, and function at low light intensities, being saturated at

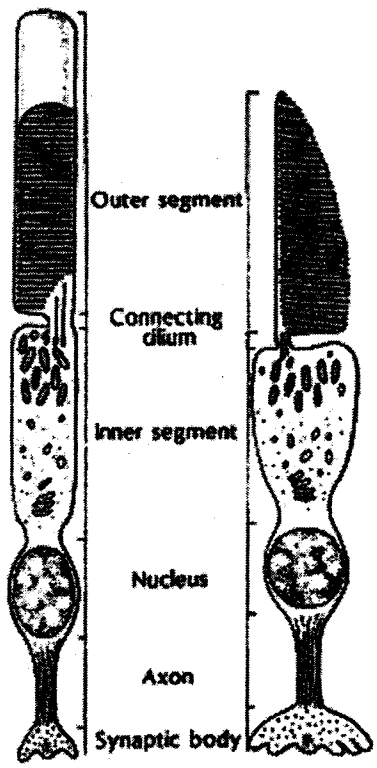

Figure 1.2. Schematic representation of rod and cone cell morphology

high intensities of light. Cones are able to function in bright light, never saturate, and provide color discrimination. While the major steps of phototransduction are similar in rods and cones, morphological, physiological and biochemical differences exist between rods and cones. At the morphological level, rods are usually longer than cones, and have a rod-like shape. They are composed of distinct stacks of membranous disks surrounded by plasma membrane. Cones, on the other hand, are generally shorter, having a cone-like shape, and are formed by invaginations of the plasma membrane (see Fig. 1.2.).

All the components of visual transduction are localized in the outer segment of the photoreceptor cells, attached to the disk membranes. Some proteins are transmembrane proteins (rhodopsin, peripherin, etc.), other are attached to the membrane via isoprenyl groups (transducin γ, PDE α and β subunits), while others are fatty acylated (transducin α, recoverin, etc.). The attachment of the isoprenylated proteins to the disk membrane is believed to be facilitated by the prenyl-binding protein PrBP-δ. The structure of rod disk membranes is maintained by distinct proteins which connect the disk rims to the plasma membrane (peripherin (Molday et al., 1987), GARP2 (Poetsch et al., 2001)).

The visual pigment (rhodopsin for rods and cone opsins for cones) is a seven-transmembrane segment receptor that resides in the outer segment membranes. The high concentration of the pigment in these structures permits a high quantum efficiency of photon capture. Physiologically, rods manifest a very sensitive response, being able to detect single photons, while cones are less sensitive, generating electrical responses only at higher intensities of light. Rods cannot however, effectively operate at high light

intensities, whereas cones are able to generate a response even at the brightest light intensities.

While the major phototransduction pathways are similar in rods and cones, the differences in sensitivity and response kinetics are due to biochemical components of the two photoreceptor cells. Rods and cones contain either different isoforms of the same protein, or proteins that are unique to a specific photoreceptor type.

2. Activation Pathway of Visual Transduction.

The main phototransduction pathway serves to regulate cGMP levels in the outer segment. Activation of the phototransduction pathway results in a decrease of the cGMP concentration in the outer segment of the photoreceptor. The activation process starts with the absorption of a photon by the visual receptor, rhodopsin. Rhodopsin is a seven

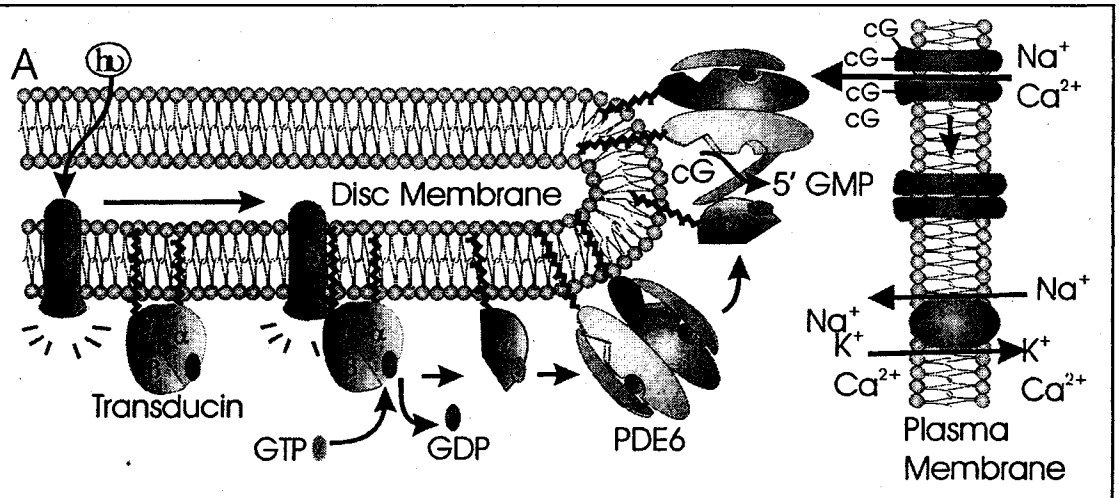

Figure 1.3. Activation pathway of visual transduction.
Components of the visual excitation pathway are bound to the disk membranes. Light activates rhodopsin (R), which will activate transducin by exchanging GDP for GTP. The dissociated Tα will interact with Pγ activating PDE6. The resulting drop in cGMP concentration will determine the closure of the plasma membrane ion channels. The $Na^+/K^+/Ca^{2+}$ exchanger will pump Ca^{2+} out of the cell, causing a drop in the Ca^{2+} concentration as well.

transmembrane protein, found in the outer segment disks. Upon light absorption, the chromophore of the rhodopsin molecule (11-cis-retinal) is isomerized to all-trans-retinal. This isomerization activates rhodopsin, which in turn activates the G-protein transducin. Transducin is a heterotrimeric G protein. Its α-subunit (Tα) binds guanine nucleotides and is anchored to the membrane by fatty acylation. The isoprenyl moiety on the γ subunit further anchores the tightly associated βγ dimer (Tβγ) to the membrane. Upon binding of Tαβγ to light-activated rhodopsin, the GDP bound to the α subunit is exchanged for GTP. With GTP bound, Tα dissociates from Tβγ and interacts with the inhibitory γ subunit (Pγ) of the photoreceptor phosphodiesterase (PDE6). The inhibitory constraint of Pγ is released upon interaction with activated Tα, such that PDE6 catalytic activity is greatly accelerated. With the catalytic activity enhanced, PDE6 rapidly converts cGMP to GMP, lowering the cGMP concentration inside the outer segment of the photoreceptor. The decrease in intracellular cGMP concentration causes cGMP dissociation from regulatory sites of the cGMP-gated channels on the plasma membrane. This in turn causes the closure of the channels. This closing of the ion channels blocks the flux of ions into the outer segment, and the plasma membrane becomes hyperpolarized (see Fig.1.3). This hyperpolarization is transmitted along the plasma membrane from the outer segment to the synaptic terminus of the photoreceptor neuron, where the signal is transmitted to other retinal neurons.

3. Inactivation of Visual Excitation.

The inactivation of the visual transduction cascade is not the reverse of its

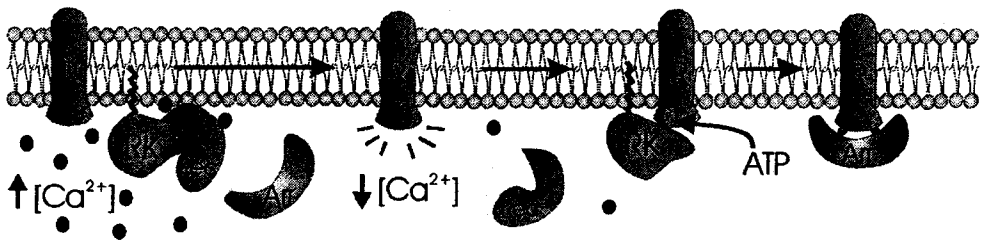

Figure 1.4. The inactivation pathway of rhodopsin (R).
The decrease in Ca2+ concentration determines the release of rhodopsin kinase (Rk) form recoverin (Rec), and phosphorylation of rhodopsin, which will allow the binding of arrestin (Arr) and inhibition of activated rhodopsin.

activation. A different set of regulatory proteins control the events of inactivation. In order for the photoreceptor to detect changes in the ambient light, it needs to rapidly return to the non-activated physiological state in order to process another set of absorbed photons. Therefore each of the activation steps is inactivated by its own set of processes. Activated rhodopsin is rapidly phosphorylated by rhodopsin kinase (Mendez et al., 2000; Kennedy et al., 2001; Ohguro, 2000; Ohguro et al., 1996) which allows arrestin to bind with high affinity and inactivate it (Vishnivetskiy et al., 1999; Gurevich and Gurevich, 2004; Krupnick and Benovic, 1998; Wilden, 1995). Arrestin binding to rhodopsin disrupts the interaction that rhodopsin makes with transducin (see Fig.1.4.).

Transducin has its own inactivation pathway, independent of rhodopsin inactivation. It is inactivated when bound GTP is hydrolyzed to GDP. The α subunit of

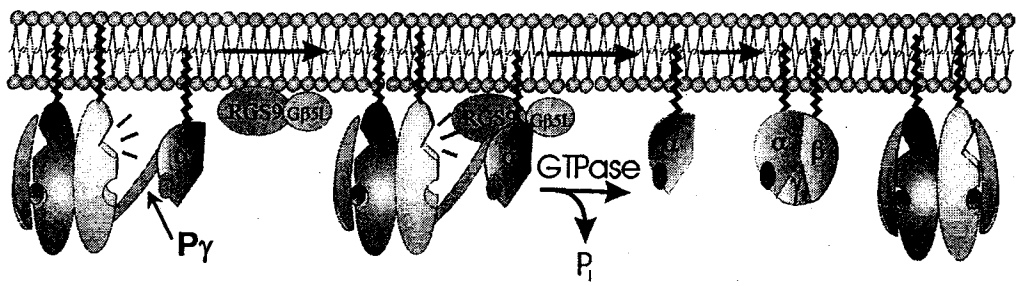

Figure 1.5. Inactivation of transducin.
Bound GTP is hydrolyzed to GDP by the intrinsic GTPase activity of Tα enhanced by the GAP proteins RGS9, Gβ5L and Pγ.

transducin has an intrinsic GTPase activity (Fung et al., 1981), but the rate of hydrolysis is slow (Sitaramayya and Liebman, 1983). To speed the rate of transducin inactivation, GTPase accelerating proteins interact with Tα (see Fig.1.5.). The protein complex that accelerates the hydrolysis rate of GTP is composed of RGS9 (regulator of G protein signaling protein 9), Gβ5 (He et al., 1998; Makino et al., 1999; Slep et al., 2001), the transmembrane protein R9AP [not shown in Fig. 1.5.; (Hu and Wensel, 2002)], as well as the inhibitory Pγ subunit of PDE6 (Slepak et al., 1995; Cote et al., 1994). The transducin-activated PDE6 is inhibited only after Tα hydrolyses its bound GTP and reforms the transducin heterotrimer. The inhibitory Pγ subunit is then able to restore the inhibited state of the PDE6 catalytic subunits.

So far, there is no known mechanism that directly inactivates PDE6, and is independent of transducin inactivation. It is possible that other mechanisms control PDE6 activity at the level of Pγ inhibition. For example, it is known that Pγ can be

phosphorylated (Paglia et al., 2002; Udovichenko et al., 1994; Xu et al., 1998) and ADP-ribosilated (Bondarenko et al., 1997; Bondarenko et al., 1999). Both modifications could directly influence PDE6 catalytic activity. PDE6 activity is also indirectly influenced by Ca^{2+} regulatory mechanisms (discussed below) that control intracellular cGMP levels. Finally, there is physiological evidence that novel proteins or pathways may regulate PDE6. A major portion of the thesis is devoted to exploring a novel regulatory mechanism involving GARP2.

4. Biochemistry of Recovery and Adaptation (Ca^{2+}, cGMP).

As described above, light activation causes a decrease in cGMP. The termination reactions restore the inhibited state of PDE6, and lead to the recovery of cGMP levels. For the restoration of cGMP levels, a new set of events takes place. The enzyme responsible for restoring cGMP level is guanylate cyclase (GC). The activity of this enzyme is regulated by Ca^{2+} levels in the outer segment. As stated earlier, light activation causes closure of the cGMP-gated channels in the plasma membrane, preventing the entry of both Na^{+} and Ca^{2+} ions inside the outer segment of the photoreceptor. At the same time Ca^{2+} is being transported out of the cell through the $Na^{+}/Ca^{2+}/K^{+}$ exchanger located on the plasma membrane (Hodgkin et al., 1987). The resulting low intracellular Ca^{2+} concentration causes dissociation of Ca^{2+} from Ca^{2+} binding protein GCAP (guanylate cyclase activating protein) which stimulates guanylate cyclase activity (Palczewski et al., 2004). By elevating the cGMP levels, the dark-adapted state of the photoreceptor is rapidly restored.

The synthesis of cGMP by guanylate cyclases also contributes to desensitizing the photoreceptor cell in response to constant illumination conditions. Photoreceptor cells need to function over a wide range of light intensities. To generate an electrical response to an incremental change in illumination intensity, photoreceptors must adapt their light sensitivity. A major regulator of light adaptation is the intracellular messenger Ca^{2+}. One mechanism of adaptation mediated by Ca^{2+} is the regulation of guanylate cyclase activity by GCAPs during extended illumination. The combination of PDE6 activation and GC activation causes an increase in cGMP metabolic flux, which represents one mechanism for light adaptation.

Another mechanism of adaptation which is controlled by Ca^{2+} concentration is the activation of rhodopsin kinase. When the Ca^{2+} concentration is high, rhodopsin kinase is bound to the Ca^{2+} binding protein recoverin, and the activity of rhodopsin kinase is inhibited (Kawamura, 1993; Gorodovikova et al., 1994; Klenchin et al., 1995; Chen et al., 1995). When the Ca^{2+} concentration drops following excitation, recoverin is not longer complexed with Ca^{2+}, and is released from rhodopsin kinase (Gray-Keller et al., 1993; Koutalos and Yau, 1996). Activated rhodopsin kinase then phosphorylates rhodopsin, which in turn induces arrestin binding, and blocks the excitation pathway (Fig.1.4.). Another adaptative mechanism regulated by Ca^{2+} concentration is the affinity of the cGMP gated channel for cGMP. In a high Ca^{2+} condition, the cGMP-gated channel is bound to the calcium binding protein, calmodulin (Hsu and Molday, 1993; Nakatani et al., 1995; Bauer, 1996). The affinity of the cGMP-gated channel for cGMP is greatly increased upon the dissociation of calmodulin which occurs when the Ca^{2+} levels are low.

The increased affinity for cGMP allows the channels to stay open at lower cGMP concentrations during light adaptation.

There are numerous other mechanisms of adaptation which are currently studied. Some of these mechanisms involve photopigment bleaching (more pronounced in cones (Rodieck, 1998; Burkhardt, 1994), Ca^{2+} buffering by Ca^{2+} binding proteins (Korenbrot and Rebrik, 2002), or translocation to the inner segment of the photoreceptor during prolonged light illumination (Arshavsky, 2003).

5. Structure and Regulation of PDE6 Catalytic and Regulatory Domains.

PDE6 is one of eleven members of the super-family of cyclic nucleotide phosphodiesterases. The preferred substrate for PDE6 is cGMP. In the PDE family PDE5 and PDE9 are also cGMP hydrolyzing enzymes (Soderling and Beavo, 2000; Francis et al., 2001), while PDE2, PDE3, PDE4, PDE7 and PDE8 are prefer cAMP as substrate (Soderling and Beavo, 2000). PDE1, PDE10 and PDE11 can hydrolyze with equal efficiency both cGMP and cAMP (Soderling and Beavo, 2000) (Kakkar et al., 1999). Each PDE family has its predominant tissue location, such as brain (PDE1, PDE2, PDE7 and PDE10), heart (PDE1, PDE2, PDE3, and PDE5), liver (PDE2), intestine (PDE9), smooth muscle (PDE5), skeleton muscle (PDE7 and PDE10), thyroid gland (PDE8), spleen (PDE9), prostate (PDE11), olfactory cells (PDE1 and PDE7), adipose tissue (PDE3) (Soderling and Beavo, 2000). PDEs also differ in their regulatory mechanisms. Many are regulated by phosphorylation (PDE1, PDE3, PDE4, PDE5), other are regulated by GAF domains (named for their occurrence in cyclic GMP-regulated PDEs, Anabaena adenylyl cyclases and the *Escherichia coli* transcription factor FhlA) (PDE2, PDE5,

PDE6, PDE10 and PDE11), and also by other mechanisms. PDE6 is the only PDE that is regulated by another subunit, the inhibitory Pγ subunit (Hurley and Stryer, 1982).

Rod PDE6 is a heterotetramer, composed of two catalytic subunits (α and β), and two identical inhibitory subunits (Pγ) (Fig.1.6.(Hurley and Stryer, 1982; Fung et al., 1990; Artemyev et al., 1996b; Cote, 2003; Kajimura et al., 2002; Kameni Tcheudji et al., 2001; Li et al., 1990).

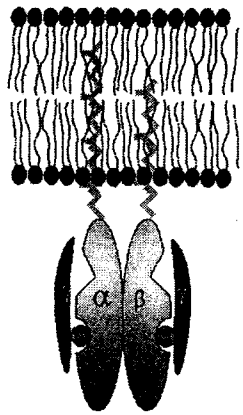

Figure 1.6. Structure of the rod PDE6.
The catalytic heterodimer is composed of the α and β subunits, each of which contains a catalytic domain (notch) and a cGMP-binding regulatory GAF domain. The activity of PDE6 is inhibited by two identical

Cone PDE6 is also believed to be a tetramer, but with two identical catalytic subunits (α') and two identical inhibitory subunits (Pγ') (Hurwitz et al., 1985; Gillespie and Beavo, 1988).

The catalytic subunits of PDE6 are composed of two domains: a regulatory domain and the catalytic domain. The regulatory domain is located in the N-terminal part of the molecule, and is composed of two tandem GAF domains, GAFa and GAFb, one of which binds cGMP. There are four potential cGMP binding sites within the GAF

domains of the PDE6 catalytic dimer. However, only two of these sites bind cGMP, each with differing affinity depending on the state of activation of PDE6 (Gillespie and Beavo, 1989; Cote et al., 1994; Mou et al., 1999). The GAFa domain is the primary dimerization site for the PDE6 catalytic subunits (Kameni Tcheudji et al., 2001; Muradov et al., 2003). Also, one site of interaction of the inhibitory Pγ subunit is located on the vicinity of GAFa domain (Muradov et al., 2004), such that GAFa regulates the catalytic activity of PDE6 through Pγ. A new study demonstrates that one Pγ binds asymmetrically to the PDEαβ catalytic dimer, at two sites at the GAF domains of the PDEαβ dimer (Guo et al., 2005). One site of interaction is located at the GAF domain of the β catalytic subunit, while the other is located at the GAF domain level of the α subunit of PDE6.

The catalytic domain of PDE6 has a strong sequence similarity with the catalytic domain of PDE5 (McAllister-Lucas et al., 1993). However, the catalytic efficiency is 1000-fold greater for PDE6 (Granovsky and Artemyev, 2001) than it is for PDE5 which is three orders of magnitude lower (Thomas et al., 1990). The catalytic pocket contains divalent cations, with a high affinity binding site for Zn^{2+} for both PDE5 and PDE6 (Francis et al., 2000; Francis et al., 1994; He et al., 2000a). Most pharmacological inhibitors of PDE5 are also effective inhibitors for PDE6 (Corbin and Francis, 2002; Cote, 2004; Estrade et al., 1998; Luke et al., 2005; Marmor and Kessler, 1999; Zhang et al., 2005b). The activation mechanism is different however for PDE6 and PDE5. While PDE6 is activated by displacement of the inhibitory Pγ subunit (described above), PDE5 is activated by phosphorylation and allosteric regulation through its GAF domains (Corbin et al., 2000; Rybalkin et al., 2003). The C-terminus of the catalytic subunit is

isoprenylated, a feature unique to PDE6 that allows it to be membrane-bound (Ovchinnikov et al., 1987; Lipkin et al., 1990; Li et al., 1990).

6. PDE6 Regulatory Proteins.

Other members of the PDE super-family are regulated allosterically through their regulatory domains. For PDE6, the primary way of regulating the catalytic activity is through interacting proteins.

The primary mean to regulate the catalytic activity of PDE6 results from the interaction with its inhibitory Pγ subunit. Pγ is an 87 amino-acid protein (Ovchinnikov et al., 1986; Hamilton and Hurley, 1990) which has the primary role in blocking the access to the catalytic site (Granovsky et al., 1997). Pγ is classified as a natively unfolded protein (Berger et al., 1997; Uversky, 2002). This is conferred by a limited secondary structure (Slep et al., 2001), which allows the Pγ molecule to span distant parts of the

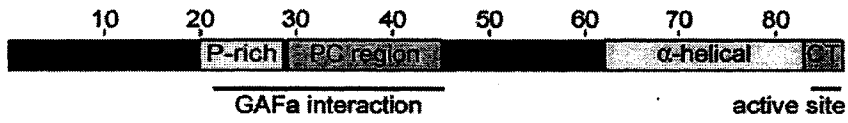

Figure 1.7. Structure of the inhibitory Pγ subunit.
In the C-terminal part is the proline-rich region and poly-cationic (PC) region which interact with the GAFa domain on PDE6. The N-terminal part interacts with the PDE6 catalytic site (CT) and has a a-helical structure

catalytic subunit (Guo et al., 2005). To effectively inhibit PDE6 catalysis, Pγ binds to PDE6 catalytic subunit at multiple sites. The highest binding affinity site of Pγ to PDE6 catalytic subunit is located in the vicinity of the GAFa domain of PDE6 (Natochin and

Artemyev, 1996). Pγ has a complex structure (see Fig.1.7.): the central region (a.a. 29-45) contains a poly-cationic motif which serves as the high-affinity site of interaction with activated transducin (Artemyev et al., 1992; Lipkin et al., 1988; Skiba et al., 1995; Morrison et al., 1989; Brown, 1992). In the N-terminal region, Pγ also contains a proline-rich region (a.a. 20-28) which may serve as the site of interaction with SH3 domain-containing proteins (Morin et al., 2003). The region of a.a. 20-45 also constitutes the major interaction site with the GAFa domain of the catalytic subunit of PDE6 (Artemyev et al., 1992; Lipkin et al., 1993; Natochin and Artemyev, 1996; Takemoto et al., 1992; Mou and Cote, 2001). The Pγ subunit also has two phosphorylation sites at a.a. T22 and T35 (Paglia et al., 2002; Udovichenko et al., 1993; Xu et al., 1998). The C-terminal region also includes a site of interaction with transducin α (Tα).

The major site of interaction of Pγ with the catalytic site resides in the extreme C-terminus part (a.a. 83-87) (Brown, 1992; Lipkin et al., 1988). Upon transducin activation, the inhibitory constraint of the C-terminal region of Pγ on the catalytic site is released by Tα-GTP interacting with this region (Artemyev et al., 1992; Skiba et al., 1996). Pγ dissociates from PDEαβ upon prolonged light activation. Upon dissociation Pγ has a GTP-ase accelerating function, and together with RGS9 and Gβ5-L regulates the GTP-ase function of Tα (Arshavsky and Pugh, Jr., 1998; He et al., 2000b; Skiba et al., 1999). Pγ also undergoes ADP-ribosylation at the central region (Arg^{33} and Arg^{36}) when it is free or in complex with PDEαβ dimer (Bondarenko et al., 1997; Bondarenko et al., 1999). However, ADP-ribosylated Pγ can not make interaction with activated transducin (Bondarenko et al., 1999).

Another PDE6 interacting protein, the Prenyl-Binding Protein (PrBP-δ) is believed to have a role in the targeting of the isoprenylated PDE6 to the disk membrane (see Fig.1.8.) (Norton et al., 2005; Zhang et al., 2004). This protein was initially thought to be a subunit of PDE6 because it co-purified with the soluble PDE6 (Florio et al., 1996; Gillespie et al., 1989), but due to its low stoichiometry of binding to PDE6, it is not considered a bona fide PDE6 subunit (Norton et al., 2005).

PrBP-δ has an immunoglobuline-like domain that forms a hydrophobic pocket (Hanzal-Bayer et al., 2002). This pocket is able to bind to isoprenyl moieties and solubilizes membrane bound protein (Cook et al., 2000; Marzesco et al., 1998; Zhang et al., 2004).

In photoreceptor cells, PrBP-δ also interacts with rhodopsin kinases, small GTPases such as Rab13, Ras, Rap, and Rho6 (Zhang et al., 2005a). It is hypothesized that

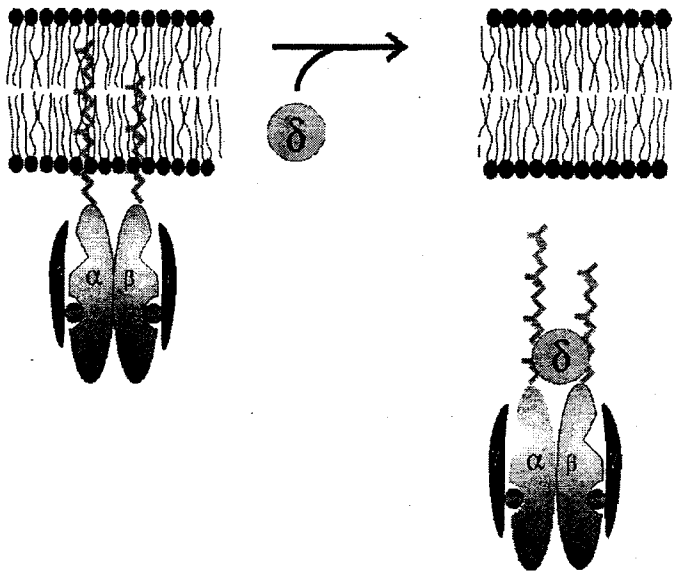

Figure 1.8. Solubilization of PDE6 by PrBP-δ in vitro. PrBP-δ binds to the isoprenyl moieties of the catalytic subunits of PDE6 releasing it from the disk membranes.

PrBP-δ is involved in the transport and membrane targeting of isoprenylated proteins from their site of synthesis to the disk membranes of the outer segment (Norton et al., 2005).

7. Structure, Localization and Role of GARP Proteins in Photoreceptor Cells.

The outer segment of rod photoreceptor cells contain a set of proteins that are not found in cones. These proteins belong to the glutamic acid-rich protein family (GARPs).

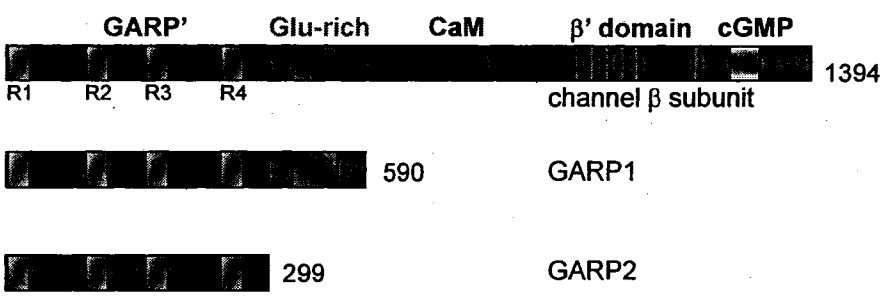

Figure 1.9. Structure of GARP proteins.
The N-terminal region contains the proline-rich repeats (R1-R4); GARP1 and the β-subunit of the channel also have the glutamate-rich domains (Glu-rich). The β-subunit of the channel has other motifs such as a calmodulin binding domain (CaM), transmembrane domains, (β' domain) and a cGMP binding domain.

The GARP family is composed of three proteins: two soluble proteins: GARP1 and GARP2, and the N-terminal part of the β-subunit of the cGMP-gated plasma membrane channel (see Fig.1.9.) (Ardell et al., 1995; Korschen et al., 1995; Sugimoto et al., 1991). The GARP part of the cGMP gated channel faces the cytoplasm of the outer segment (Colville and Molday, 1996). All three members of the GARP family are splice variants of the same gene (Ardell et al., 1995). All three GARP proteins exhibit abnormal migration of SDS-PAGE. It is believed that this is due to the high content of glutamate residues which diminish the SDS binding to the protein (Korschen et al., 1995).

The amino-acid sequence of the three GARP proteins reveals some common features. The first 291 amino-acids of all the GARP proteins are identical (Ardell et al., 1996; Colville and Molday, 1996). The N-terminal region contains four proline-rich repeats, which are also the most conserved regions among GARPs from different species (Ardell et al., 1996; Colville and Molday, 1996; Korschen et al., 1995; Sugimoto et al., 1991). One early hypothesis was that these proline-rich repeats favored protein-protein interactions with PDE6, peripherin, guanylate cyclase, the α-subunit of the channel and GARPs (Korschen et al., 1999). Further studies demonstrated however, that some of the above-mentioned proteins are not interacting with the full-length GARPs, and only peripherin, and other GARPs are binding to GARPs (Poetsch et al., 2001). Another characteristic of the glutamic acid-rich proteins is the presence of the glutamic acid-rich motif. However, this motif is present only in the β subunit of the cGMP-gated channel and in GARP1; although GARP2 lacks this motif, the content of glutamate residues is still high throughout the protein (Colville and Molday, 1996).

The last eight C-terminal amino-acids of GARP2 are unique, which serves as a means to generate specific antibodies to GARP2 (Colville and Molday, 1996). Immunolocalization studies with GARP2-specific antibodies show that it is localized primarily at the rim of the outer segment disks, in close proximity with the plasma membrane (Colville and Molday, 1996; Korschen et al., 1999). It has been proposed that GARP2 serves as a structural bridge between the plasma membrane of the rod photoreceptor and the disks (Poetsch et al., 2001). The sequence analysis of the GARP proteins reveals another characteristic: the presence in high abundance of proline-

glutamate-serine-threonine rich domains. These so-called PEST sequences are associated with rapid degradation of the protein due to proteolysis (Rechsteiner and Rogers, 1996). Recent study showed that GARP2 belongs to the family of "natively unfolded" proteins, lacking a high degree of complexity of the secondary structure (Batra-Safferling et al., 2006). This characteristic is usually associated with proteins that serve as scaffolds, and GARP2 might serve as a structural protein preserving the integrity of the rod outer segment (Batra-Safferling et al., 2006).

Another proposed role of GARP2 is as a regulator of PDE6 activity. Korschen et al. (1999) showed that GARP2 is able to inhibit the activity of transducin-activated PDE6, but has no effect on trypsin-activated or non-activated PDE6 activity. This finding would suggest that GARP2 might serve as alternative mechanism of adaptive regulation of the photoreceptor cells.

8. Hypotheses to be Tested.

The overall objective of this thesis is to identify and characterize alternative mechanisms for regulation of PDE6 activity.

It is hypothesized that PDE6 is regulated by other mechanisms in addition to the classical excitation pathway. These interactions may modulate PDE6 activity during later stages of the visual process. The proteins that interact with PDE6 (Tα, Pγ) have shown a regulatory effect on its catalytic activity. However, the rod photoreceptor contains other proteins, some of which are present in low abundance, that might interact with PDE6 and have an effect on the phototransduction pathway.

The first goal of this research is to determine the PDE6 "interactome". The different states of PDE6 activation may cause different proteins to associate and dissociate. Therefore, each activation condition needs to be studied for the interacting partners of PDE6. Once all of the binding partners for nonactivated and activated forms of PDE6 are identified, the role of each individual protein can be determined.

A second major goal of this thesis is to more fully characterize the PDE6 interacting protein GARP2. A previous study showed that GARP2 interacts with PDE6 and reportedly regulates PDE6 in its transducin-activated state (Korschen et al., 1999). This finding would suggest a role for GARP2 in desensitizing the visual excitation pathway during bright light. This function of GARP2 to regulate PDE6 would only be relevant to rod photoreceptors, since GARP2 is absent in cone photoreceptors. One important difference in rod and cone photoresponse is their light sensitivity. Rods are very sensitive, being able to respond to a single photon of light, while cones function only at higher intensities of light. The higher sensitivity of rods results from a very low dark "noise" of the photoreceptor, compared to cones that have a much higher background "noise". My objective is to assess whether GARP2 regulates rod photoreceptor activity in a way that would account for the different physiology of rods versus cones.

In order to assess the physiological and biochemical significance of GARP2 binding to PDE6, it is necessary to determine the total amount of GARP2 in the outer segment of rod photoreceptors. If GARP2 is present in sufficient amounts relative to PDE6, then it is capable of regulating the entire pool of PDE6 during visual transduction.

We also had to determine how GARP2 binds and regulates PDE6 catalytic activity, and what are the consequences of GARP2 binding to PDE6 on the activation-inactivation cycle of PDE6.

The second goal is to study the interaction of GARP2 with PDE6, and the effect that GARP2 has on the PDE6 catalysis. In order to accurately determine GARP2 function, a method to obtain purified native GARP2 isolated from the photoreceptor cells is required. One limitation of the Korschen et al study (1999) is the use of recombinant GARP2 fusion protein, and the assumption that the tag does not influence the regulatory properties of PDE6. In this thesis I describe several approaches for purifying native GARP2 free of PDE6 subunits.

CHAPTER 2

ISOLATION OF PDE6 INTERACTING PROTEIN COMPLEX AND IDENTIFICATION OF ITS COMPONENTS. STRATEGIES FOR PDE6 PURIFICATION.[2]

Abstract

Rod photoreceptor phosphodiesterase (PDE6)[3] is the only phosphodiesterase that is membrane associated through isoprenyl motifs at the C-terminal region of the catalytic subunits. In order to study the PDE6 interactions with other proteins during the visual transduction cascade, an effective solubilization method needed to be developed. We found that 10 mM of the zwitterionic detergent CHAPS is able to solubilize the majority

[2] Parts of this study were published in Methods in Molecular Biology: Pentia DC, Hosier S, Collupy RA, Valeriani BA, Cote RH., 2005;307:125-40.

[3] The abbreviations used are: PDE6, photoreceptor phosphodiesterase; Pγ, inhibitory 10 kDa γ subunit of PDE6; GARP2, glutamic acid-rich protein 2; PrBP-δ, 17 kDa prenyl-binding protein; GST, glutathione S-transferase; GTPγS, guanosine 5'-3-O-(thio)-triphosphate; ROS, rod outer segment; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; CHAPS, 3-(3-Cholamidopropyl)dimethylammonio)-1-propanesulfonate; BCA, bicinchoninic acid.

of PDE6 from the ROS membranes, and in the same time the ability of transducin to activate solubilized PDE6 is not altered.

The second aim is to evaluate the light regulation of protein interactions with PDE6. The different stages of light activation are induced by the use of nucleotides, GTPγS for permanent activation of transducin, cGMP for the occupancy of the GAF domains of PDE6, and ATP for protein phosphorylation. In the same time, various solubilization methods are used for the analysis of the proteins that interact with PDE6. We found that upon PrBP-δ solubilization, GARP2 is released from the PDE6 complex. Also, PDE6 complex runs at higher than expected apparent molecular masses on size exclusion chromatography, suggesting the presence of interacting proteins.

Based on the findings of PDE6 interacting proteins under different activation conditions and the analysis of various solubilization methods, we developed an improved method to purify PDE6 holoenzyme devoid of binding partners. A procedure to purify PDEαβ heterodimer is also described.

Introduction

Phosphodiesterase (PDE6) is the central effector in the phototransduction cascade in rod and cone photoreceptors. The most abundant PDE6 isoform of rod photoreceptor cells is the membrane-associated PDE6, present in the rod outer segment (ROS) portion of the cell. Although displacement of the inhibitory γ subunit of PDE6 (Pγ) by activated transducin is the primary regulator of PDE6 activation, many other factors are thought to be involved—directly or indirectly—in PDE6 regulation, including: Prenyl-binding protein –δ (PrBP-δ) (Cook et al., 2001; Mou et al., 1999; Norton et al., 2005), glutamic acid-rich protein 2 (GARP2) (Korschen et al., 1999), regulator of G protein signaling 9 (RGS9) (He et al., 1998), and the Gβ5L isoform of G protein (Makino et al., 1999). We sought to investigate potential protein-protein interactions between PDE6 and other regulatory proteins, and how these interactions might be affected by light activation.

The study of proteins that interact with PDE6 during phototransduction is hampered by the membrane-association of rod photoreceptor PDE6 on the disk membrane. In addition, ROS membranes have been shown to be critical for efficient activation of phototransduction (Malinski and Wensel, 1992; Melia et al., 2000). Therefore, the optimal solubilization procedure for PDE6 that retained its activity but did not disrupt disrupting protein-protein interactions has been researched. To do this, the ability of various detergents, solubilizing proteins like PrBP-δ, and hypotonic buffers to release PDE6 from the membrane has been explored.

Because phototransduction is a dynamic process, regulated by the primary messenger light, the interaction between its components is also dynamic. Proteins that

take part in generating the electrical response are associating and dissociating depending on the activation or deactivation state of the interacting partners. It is important therefore to determine which proteins interact with each other during different phases of the visual transduction cascade.

In order to determine the organization of the protein complexes in dark-adapted and light-stimulated conditions, two variables have to be considered. First, the different stages of visual activation have to be induced in the photoreceptor cells. Moreover, to allow the separation of the protein complexes, the various steps of the activation process have to be halted and preserved at least for the duration of the isolation procedure. The second variable that has to be considered is the strength of interaction between proteins in the complex. Some interactions are weak, and of short duration, such that different procedures have to be used. Both of these considerations are addressed in the present study.

In order to control the activation states of PDE6, the factors that regulate the formation of each activation step need to be understood and controlled. PDE6 undergoes different states of activation-inactivation during the phototransduction cycle that involve primarily in the release and re-attachment of the inhibitory Pγ subunit (Hurley and Stryer, 1982). But the PDE6 holoenzyme can also be regulated by the binding of the cGMP to the GAF domains (Yamazaki et al., 1982; Yamazaki et al., 1996; Cote et al., 1994; Mou et al., 1999). This confers a means to regulate the activation state of PDE6 based on the amount of cGMP present. Also the activation state of PDE6 is dependent on the activation of transducin (Bennett and Clerc, 1989; Fung and Nash, 1983; Malinski and Wensel, 1992; Otto-Bruc et al., 1993; Tyminski and O'Brien, 1984). Transducin gets

activated by rhodopsin, and exchanges bound GDP for GTP. By controlling the availability of GTP, it is possible to vary the extent of transducin activation. During de-activation however, GTP is hydrolyzed to GDP by the intrinsic GTPase activity of transducin (Fung et al., 1981), such that activated transducin state is transient. To induce persistent transducin activation, use of the non-hydrolyzable GTP analog GTPγS is required. Some proteins involved in visual transduction (which directly or indirectly influence PDE6 activation), are regulated by phosphorylation. One PDE6 subunit that is thought to be phosphorylated under different activation states is Pγ (Hayashi, 1994; Hayashi et al., 1991; Hayashi et al., 2000; Matsuura et al., 2000; Paglia et al., 2002; Tsuboi et al., 1994a; Tsuboi et al., 1994b; Udovichenko et al., 1994; Xu et al., 1998). There is also evidence that the catalytic dimer of PDE6 undergoes phosphorylation (Udovichenko et al., 1993). Different states of those proteins could be controlled by the addition of ATP as a substrate for protein kinases. Overall, the various stages of PDE6 activation-deactivation can be "frozen" by addition or omission of different nucleotides that control various steps in the phototransduction pathway.

Another factor that needs to be considered when studying changes in protein association during phototransduction is the ability to preserve protein interactions during isolation of the protein complexes. Because some proteins are attached to the disk membranes, and some interactions might be low affinity, the appropriate method needs to be considered to extract the complex and preserve those interactions. In the present study several solubilization methods are used, and compared with regard to the complex of proteins that each method is isolating. Three methods have been used in this study:

solubilization of the PDE6 protein complex using a hypotonic solution, using mild detergent, or using the isoprenyl-binding protein PrBP-δ.

In this study, we examine the PDE6 interacting proteins under different activation states, using previously optimized methods of solubilization for PDE6 (Maftei, 2000).

Materials and Methods

1. Materials:

Bovine retinas were purchased from W. Lawson (Lincoln, NE). Chromatography columns and media (Mono Q and Superdex prepacked columns, butyl-Sepharose, and Q-Sepharose) were from GE-Healthcare, ceramic hydroxyapatite type I from Bio-Rad Laboratories. Sulfolink coupling gel and Immunopure (G) IgG Purification Kit were from Pierce. Membrane filtration devices were from Millipore. The ROS1 monoclonal antibody cell line was a kind gift from Dr. R. L.Hurwitz (Baylor College of Medicine).

Miscellaneous stock solutions were prepared as follows; 1 M DTT in water, 100 mM PMSF in 95% ethanol, mammalian protease inhibitor cocktail (P8340; Sigma Chemical Corporation) as directed by manufacturer.

All solutions used to isolate and purify PDE6 are supplemented just before use with 1 mM dithiothreitol (DTT) and 0.3 mM phenylmethylsulfonyl fluoride (PMSF) or protease inhibitor cocktail (following the manufacturer's recommendations). All chromatography buffers were filtered with a 0.45-μm membrane under vacuum immediately before use to remove particulates and degas the solvent.

Antibodies: anti-noncatalytic domain of PDE$\alpha\beta$, from UNH; anti-transducin α, from Affinity bioreagents; anti-C-terminal region of Pγ, from UNH; anti-GARP2, a kind gift of Dr. Benjamin Kaupp (Julich, Germany).

2. Initial isolation of PDE6 isoforms from bovine retina and purification of ROS.

Mechanical disruption of photoreceptor cells from the neural retina was the starting point for isolating both the rod photoreceptor PDE6 associated with the outer segment membranes and the soluble rod and cone PDE6 that were recovered in the soluble portion of the retinal extract. For the case of the membrane-associated PDE6, sucrose density gradient centrifugation results in purified ROS in which rhodopsin (~70% of total protein), transducin (~10% of total protein), and PDE6 (1 to 2% of total protein) are membrane-bound. To prevent activation of the components of the phototransduction pathway, the following procedures were performed in a darkroom with infrared (IR) illumination and IR viewers. All solutions were ice-cold throughout the ROS purification process.

50 frozen bovine retinas were quickly thawed and kept on ice once thawed. 45 mL of 45% (w/v) sucrose in solution A: (20 mM 3-morpholinopropane-1-sulfonic acid (MOPS), pH 7.2; 2.0 mM $MgCl_2$, 60 mM KCl; 30 mM NaCl supplemented with 200 µL of Mammalian Protease Inhibitor Cocktail) was added to a beaker containing the 50 retinas. A magnetic stir bar was placed in the bottom of the beaker at low speed to mechanically disrupt photoreceptors from the retinas for 1 h in the dark. The solution containing the disrupted retinas was transferred to 50 mL centrifuge tubes and centrifuged at 3000 x g for 3 min to pellet the retinal debris. The retinal extract

(containing ROS) was strained through a nylon sock into a cold beaker and was diluted 1.5-fold with solution A to dilute the sucrose concentration. The retinal extract was centrifuged in 50 mL tubes for 30 min at 23,000 x g in a fixed angle rotor. The supernatant was saved for purification of cone PDE6, and the pellet containing the ROS was further purified.

3. Purification of ROS.

The ROS-containing pellets were resuspended in 15 mL of solution B1 (51 mL of 50% sucrose in solution A are diluted to 100 mL with solution A; $\rho = 1.105$ g/mL). Just before use, discontinuous sucrose gradients were prepared as follows in 18-mL centrifuge tubes (Beckman) by layering 5 mL of solution B3 (64.5 mL of B1 diluted to 100 mL with A; $\rho = 1.135$ g/mL), then 5 mL of solution B2 (54.25 mL of B1 diluted to 100 mL with A; $\rho = 1.115$ g/mL). The resuspended ROS pellets (in solution B1) were layered within 1 cm of the top of the tubes. The sucrose gradients were centrifuged for 60 min at 116,000 x g in a swinging-bucket rotor at 4°C. The interface of solutions B2 and B3 containing the purified ROS was removed with a 15-gauge needle attached to a 5-mL syringe. The ROS was diluted with 2 vol. of solution A, and then centrifuged for 60 min at 30,000 x g to pellet the purified ROS. ROS pellets were stored at -80°C until use.

4. Preparation of ROS membranes containing PDE6.

The soluble proteins present in the ROS were removed by homogenizing (in a Dounce tissue grinder at 4°C) the ROS in an isotonic buffer (10 mM Tris, pH 7.5, 60 mM KCl, 40 mM NaCl, 0.2 mM $MgCl_2$, 1 mM DTT) in the dark. ROS membranes were

separated from the soluble proteins by centrifugation. The resulting ROS membranes contain integral membrane proteins (predominantly rhodopsin), along with peripheral membrane proteins (notably PDE6 and a reduced amount of transducin). Because transducin undergoes a light-dependent binding to photoactivated rhodopsin (Kuhn, 1982), the ROS membranes were exposed to light just before releasing PDE6R with a low ionic strength buffer (see next section).

5. Extraction of PDE6 from ROS membranes with hypotonic buffer.

The ROS membrane pellets were exposed to room light for 1 min at 4°C to photoactivate rhodopsin, thereby inducing tight binding of transducin to the ROS membranes. The light-exposed ROS membranes were resuspended in a hypotonic PDE6 extraction buffer (10 mM Tris pH 7.5, 0.2 mM $MgCl_2$, 1 mM DTT). The ROS membranes were homogenized in a Dounce tissue grinder at 4°C by 10 passes of the pestle. The hypotonic extract was centrifuged for 60 min at 30,000 x g, and the hypotonic supernatant, which contains solubilized PDE6 was recovered. The hypotonic extraction procedure (without homogenization) was repeated two additional times. Pooled hypotonic extracts were clarified by ultracentrifugation at more than 100,000 x g for 30 min.

6. Purification of PDE6 by Mono Q anion-exchange chromatography.

The hypotonic PDE6 solution was adjusted to the approximate ionic strength of MQ-A buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$) by adding 0.10 vol. of MQ-B buffer (MQ-A containing 1.0 M NaCl). The solution was filtered with a

low-protein binding 0.22-μm filter to remove particulates. The PDE6 sample was loaded onto the Mono Q column at a flow rate of 0.5 mL/min and the column was washed with 5 column vol. of MQ-A buffer. A linear salt gradient from 0% MQ-B (100 mM NaCl) to 100% B (1.0 M NaCl) in a total volume of 40 mL was performed, and 1-mL fractions were collected. After the elution was completed, the Mono Q column was washed with 5 column vol. of MQ-B, and then stored as directed by the manufacturer. Fractions containing PDE6 were identified by a colorimetric PDE activity assay (Cote, 2000).

7. Ultrafiltration of PDE6 sample.

A centrifugal filter device (Centricon Plus-20; Millipore) with molecular weight cut-off of 30 kDa was used to concentrate the PDE6 sample. The sample was loaded on top of the filter, and spun in a centrifuge at 3500 x g. The concentrated sample was recovered from the top of the filter, and the flow-through was discarded.

8. Gel filtration chromatography of PDE6.

The PDE6 sample volume was reduced by ultrafiltration to $\leq 2\%$ of the total volume of the gel filtration column in order to obtain maximum resolution of the PDE6 peak. The gel filtration column was equilibrated with 2 column vol. of GFC buffer (10 mM Tris-HCl, pH 7.5, 300 mM NaCl, 2 mM $MgCl_2$) prior to injecting the PDE6 sample on the column. The column was operated at a flow rate of 0.4 mL/min and 0.3-mL fractions were collected. The absorbance at 280 nm was monitored, and the PDE6 catalytic activity of each fraction was analyzed.

9. Calibration of the Superdex-200 gel filtration column.

The following globular proteins were used to calibrate the Superdex-200 column: aldolase (10 mg/ml), catalase (10 mg/ml), ferritin (1 mg/ml), thyrodlobuline (10 mg/ml), and carbonic anhydrase (10 mg/ml). The void volume was measured with the use of Blue-Dextran (1 mg/ml). 500 μL of each protein solution was injected individually, and was eluted with GFC buffer. The OD_{280} was monitored throughout the run to detect the protein peak.

10. Immunoprecipitation with ROS1 antibody.

The murine monoclonal antibody ROS1 was originally raised against the PDE6R holoenzyme (Hurwitz et al., 1984). The epitope recognized by the ROS1 antibody has not been defined, but the affinity of the antibody for PDE6R and PDE6C is very high (Hurwitz et al., 1984).

10.1. Coupling of ROS1 antibody to sulfolink beads.

ROS1 antibody was purified from ascites fluid on a Pierce Immunopure Protein G agarose column using the manufacturer's protocol. The eluted ROS1 antibody was concentrated to less than 1 mL using a Centricon Plus-20, and diluted 10-fold with the coupling buffer, and reconcentrated to $\geq$10 mg/mL in a volume of $\leq$ 2.5 mL. ROS1 antibody was then coupled to the Sulfolink beads at a coupling density of 5 mg of antibody/mL of resin following the manufacturer's instructions.

10.2. Purification of PDE6 by adsorption to ROS1-Sulfolink column and elution of active enzyme.

ROS1-Sulfolink column was washed with 10 column vol. of pH 10.8 elution buffer (25 mM 3-cyclohexylaminopropane-1-sulfonic acid (CAPS), 200 mM NaCl, 2 mM $MgCl_2$, 10% glycerol, pH 10.8) at 1 mL/min, and then equilibrated with 10 column vol. of TMN buffer (50 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.5 mM $MgCl_2$). PDE6 sample was diluted in TMN buffer to adjust the pH and the ionic strength. PDE6 solution was loaded onto the column at a flow rate of 0.5 mL/min.The eluate is recovered. The beads were washed with 5 column volumes of TMN at 1 mL/min. Contaminating proteins that nonspecifically bind to the ROS1-Sulfolink beads were removed by washing with 5 column vol. of pH 9.0 wash buffer (25 mM CAPS, 200 mM NaCl, 2 mM $MgCl_2$, pH 9.0). PDE6 was eluted with 10 column vol. of pH 10.8 elution buffer. 1-mL fractions were collected in tubes containing 0.1 mL of neutralization buffer (1.0 M Tris-HCl, pH 6.7). The fractions are assessed for PDE activity using standard activity assays (Cote, 2000). Elution of active PDE6 from ROS1 has required extensive manipulation of buffer conditions, with high pH and the inclusion of glycerol serving to elute the antibody with the least loss of biological activity. Inclusion of a pH 9.0 wash step just prior to elution served to eliminate proteins that would otherwise contaminate PDE6 preparation.

The ROS1-Sulfolink resin is regenerated by washing with 5 column vol of pH 10.8 elution buffer, then 10 column vol. of TMN buffer containing 0.05% NaN_3.

11. Preparation of PrBP-δ.

E. coli cells containing the PrBP-δ plasmid (a kind gift of Drs. Terry Cook and Joe Beavo; Univ. of Washington) were cultured overnight in a LB medium. 10 mL of the overnight culture was inoculated in two flasks with 500 mL TY medium, and incubated at 37°C until the OD_{600} reached 0.6-0.8. At this point, protein expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside. The culture was incubated for another hour at 37°C. Cells were pelleted by centrifugation, washed, and sonicated. The supernatant containing the soluble proteins was separated from the cellular debris by centrifugation. The extracted proteins were applied to a 2 mL glutathione-agarose column. Bound GST-PrBP-δ was eluted with a solution contaning reduced glutathione, and concentrated by ultrafiltration. The protein concentration of the purified GST-PrBP-δ was determined by a BCA protein assay (Smith et al., 1985).

12. Preparation of PDEαβ heterodimer.

To determining optimal conditions for trypsin activation of PDE6, it was proteolyzed with trypsin at 4°C for various times to determine the minimum time necessary to activate the enzyme fully. After quenching the reaction with a molar excess of soybean trypsin inhibitor, samples were assayed for PDE6 catalytic activity.

PDE6 was diluted to a concentration of 200 nM in 2X proteolysis buffer (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 4 mM $MgCl_2$, 40% glycerol), and an equal volume of 100 μg/mL of TPCK-treated trypsin in 10 mM Tris-HCl (pH 7.5) was added. At various intervals, portions were removed and quenched with an equal volume of 2X proteolysis stop solution (20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 4 mM $MgCl_2$, 0.5 mg/mL of

soybean trypsin inhibitor, 0.4 mg/mL of bovine serum albumin, 0.4 mM Pefabloc, 4 mM DTT). The quenched samples were assayed for catalytic activity using a colorimetric PDE activity assay (Cote, 2000).

To prepare purified PDE$\alpha\beta$ dimer lacking Pγ or Pγ fragments, the trypsin-activated PDE6 was concentrated by ultrafiltration using a 30-kDa molecular mass cutoff filter, and resuspended in MQ-A buffer. The PDE6 was reconcentrated to remove trypsin, trypsin inhibitor, and some Pγ fragments from the PDE6 sample. Following this buffer exchange procedure, the concentrated PDE6 sample was loaded onto a Mono Q column as described above. The resulting fractions were analyzed for PDE6 activity, and concentrated by ultrafiltration to a volume of less than 500 μL and stored at –20°C with 50% glycerol. An additional gel filtration step was occasionally used to further eliminate residual Pγ fragments.

Results and Discussion

1. Analyzing PDE6 protein complexes by immunoprecipitation using various solubilization methods.

Based on previous studies (Maftei, 2000), the solubilization methods that proved to be the most effective in extracting PDE6 from the ROS membranes were either 10 mM CHAPS, an excess of PrBP-δ, or hypotonic extraction (using 5 mM Tris, pH 7.5, 0.2 mM $MgCl_2$). The zwiterionic detergent CHAPS is able to solubilize ~90% of the total PDE6 in ROS membranes when used at 10 mM concentration (Maftei, 2000). The prenyl-binding protein PrBP-δ can solubilize > 90% of the total PDE6 from the ROS membranes when used in excess over PDE6 (30 PrBP-δ per PDE6) (Norton et al., 2005). PDE6 is also released from the ROS membranes under hypotonic conditions, and ~70% of the total PDE6 can be extracted when using multiple hypotonic solubilizations (Kuhn, 1982).

PDE6 is in a dynamic equilibrium of association and dissociation with different proteins involved in visual transduction. Various activation stages involve changes in nucleotide binding and/or protein phosphorylation. Therefore, different PDE6 complexes can be induced by the use of nucleotides in conjunction with illumination. The nucleotide conditions used for inducing different activation states of PDE6 were: 1. Non-activated ROS membranes washed of any endogenous nucleotides; 2. Persistently activated ROS membranes supplemented with 1mM GTPγS; 3. Activated ROS membranes with PDE6 GAF domains occupied by including 1 mM cGMP and 1mM GTPγS; and 4. Activated ROS membranes with occupied GAF domains, and proteins in their phosphorylated state, by supplementing the ROS with 1 mM GTPγS, 1 mM cGMP and 1 mM ATP.

Following nucleotide addition and solubilization of PDE6 from the ROS membranes, the protein complex was isolated by immunoprecipitation using the anti-PDE6 specific monoclonal antibody ROS1 (Hurwitz and Beavo, 1984) coupled to agarose beads. The immunoprecipitated proteins are then separated on SDS-PAGE and transferred to nitrocellulose membranes for immunodetection. The proteins probed for were: PDEαβ, Pγ, GARP2 and transducin α (Tα).

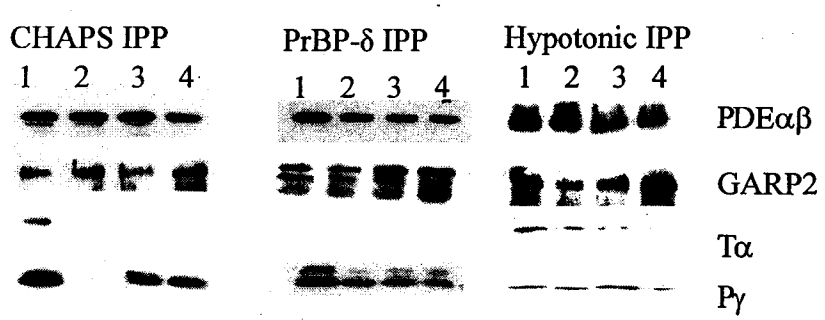

Figure 2.1. Effect of transducin activation and nucleotide addition upon the interaction of PDE6 with other peripheral membrane proteins.
ROS membranes (50 μM Rho) were treated with the following nucleotides: 1 - no addition of nucleotides; 2 – 100 μM GTPγS; 3 – 100 μM GTPγS and 1 mM cGMP; 4 – 100 μM GTPγS, 1 mM cGMP and 1 mM ATP. The three extraction methods tested are: detergent extraction using 10 mM CHAPS; PrBP-δ solubilization using 30 PrBP-δ per PDE6; hypotonic extraction using a 5 mM Tris, 0.2 mM $MgCl_2$ buffer. PDE6 extracts were immunoprecipitated with 10 μL of 5 mg/ml antibody of ROS1-Sulfolink beads. The equivalent of 1 pmol of PDE6 was loaded in each lane. The antibodies used were: the anti-catalytic PDE6 subunit, anti-full-length GARP2, anti-transducin α subunit, and anti-C-terminus of the inhibitory Pγ subunit. The GARP2 band is a false positive, being the heavy chain of the ROS1 antibody (see text below).

As seen in Fig.2.1., under all the conditions tested, Tα does not maintain an interaction with PDE6. Because upon light activation Tα has a higher affinity for activated rhodopsin on the ROS membrane, it is possible that all the solubilization methods used

destroy the interaction between Tα and PDE6, releasing only the PDE6. Another possibility is that Tα competes with ROS1 antibody for the same binding sites on PDE6. However, PDE6 is equally well immunoprecipitated in all cases. This finding would suggest that the interaction between PDE6 and Tα is of low affinity, being disrupted under the experimental conditions used.

Pγ has the strongest interaction with PDEαβ under the nonactivated condition. This is expected since PDE6 has a high affinity for Pγ in vitro. The absence of Pγ from the activated PDE6 in the absence of the GAF domains occupancy (condition 2, CHAPS extraction) correlates with the previously observed dissociation of Pγ from PDEαβ upon cGMP release from the GAF domains (Cote et al., 1994). The weaker signal for Pγ for all hypotonic conditions could suggest the weakening of the Pγ affinity for PDE6 under the hypotonic extraction conditions.

Initially we believed that GARP2 co-precipitated with PDE6 under all the nucleotide conditions used. Closer examination revealed that the band that appears at 60 kDa (the molecular weight at which GARP2 runs) is actually the immunoglobulin (IgG) heavy chain of the ROS1 antibody that dissociates from the beads upon treatment with gel sample buffer. This heavy chain reacts with the horseradish peroxidase (HRP)-conjugated secondary antibodies and appears as a band on the film. In order to detect GARP2 in the PDE6 protein complex, we employed an alternative method using a biotin-streptavidin system to detect of the proteins of interest without antibody interference. All

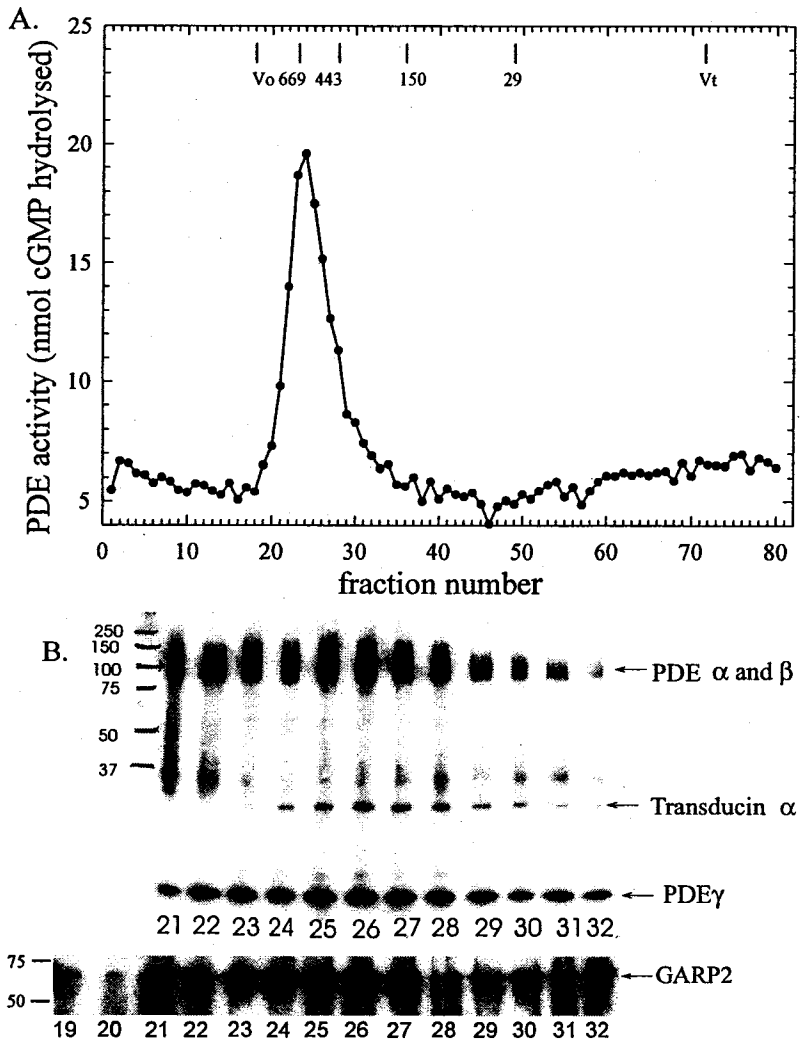

Figure 2.2. Proteins co-purifying with hypotonically extracted frog PDE6 on size exclusion chromatography.
Hypotonically extracted frog PDE6 (500 μL of 200 nM PDE6) was chromatographed on a Superdex-200 size exclusion column. **A.** 0.3 mL fractions were collected and analyzed for PDE6 activity. **B.** 20 μL of each fraction in the PDE6 activity peak were further analyzed on western blots, using anti-catalytic subunit PDE6, C-terminus Pγ, full-length GARP2 and transducin α antibodies. One blot was probed sequentially for the catalytic subunit of PDE6, Pγ and Tα, and another blot was probed for GARP2.

the antibodies used for protein detection were biotinylated, and proved to be effective in detecting proteins of interest (data not shown). Studies were not carried to further to analyze the PDE6 immunoprecipitating proteins using biotinylated antibodies.

2. PDE6 interacting proteins solubilized by hypotonic extraction.

The immunoprecipitation method has several limitations, including the likelihood of non-specific interactions with other proteins. An alternative method to isolate the PDE6 interacting complex by size exclusion chromatography was therefore examined. Size exclusion chromatography allows the separation of the protein complexes based on the size of the complexes which will elute with different relative mobilities. Analyzing the co-migrating proteins using western blotting, the identity of the proteins that form the PDE6 complex can be determined. This method also has its limitations, particularly the dilution induced dissociation of proteins that bind with low affinity which can occur during size exclusion chromatography.

Extracting proteins from membranes, in this case from rod outer segments disk membranes, was achieved by exposing the ROS membranes to a hypotonic solution. For this purpose, frog ROS membranes were used. The advantage of frog ROS over bovine ROS results from the ability to precisely control the light history of the ROS prior to retinal isolation. ROS were purified in the dark, and subsequently exposed to light for the different activation conditions during the hypotonic extraction process. The hypotonic extract was subjected to size exclusion chromatography on a Superdex-200 column, previously calibrated using globular proteins as standards (see Experimental Procedures). Following size exclusion chromatography, the PDE6 peak was identified using a colorimetric enzymatic assay (see Fig. 2.2). The apparent molecular weight of the complex was determined to be ~600kDa, almost three times greater than the predicted molecular weight of the PDE6 holoenzyme. Known interacting proteins (e.g. GARP2 (32 kDa), Tα (36 kDa)) may account in part for the larger than expected molecular weight of

the complex. Other binding partners whose identity is unknown may also contribute to the estimated molecular weight. Another factor that can contribute to the estimated molecular weight of the complex is the shape of the PDE6 complex. For example, if the protein complex has an ellipsoid shape, it will elute at apparent molecular weights greater than if the complex was a globular shape. Western blot analysis of the proteins that co-elute with PDE6 after hypotonic extraction and size exclusion chromatography reveals the presence of GARP2, the inhibitory Pγ subunit, and Tα.

3. PDE6 protein complex solubilized by detergent.

Previous work (Maftei, 2000) determined the optimal detergents and concentrations for the release of PDE6 from the ROS disk membranes. For example, 10 mM CHAPS (a zwiterionic detergent) is able to release most of the PDE6 from the membrane, while preseving transducin's ability to activate the solubilized PDE6. However, detergent solubilization may also disrupt some protein-protein interactions.

A detergent extract of PDE6 was subjected to size exclusion chromatography. Following the chromatographic run, the PDE6 peak was identified by PDE6 activity assay. We found that PDE6 elutes in two separate peaks on the Superdex-200 column (see Fig.2.3.). The first peak eluted in the void volume of the column. The second peak eluted at an apparent molecular weight of ~380 kDa. This is also a greater molecular weight than the calculated one. Analyzing proteins that elute in the second peak by western analysis, it has been observed that GARP2 is absent, but PDE6 co-elutes with PrBP-δ (data not shown). The binding of PrBP-δ to PDE6 could cause a more oblong

shape of the complex, which could influence the relative mobility of the complex, and result in a higher apparent molecular weight.

The most probable cause for the elution in the void volume is the formation of micelles by the detergent. The critical micellar concentration of CHAPS is 5 mM, half the concentration used to solubilize PDE6.

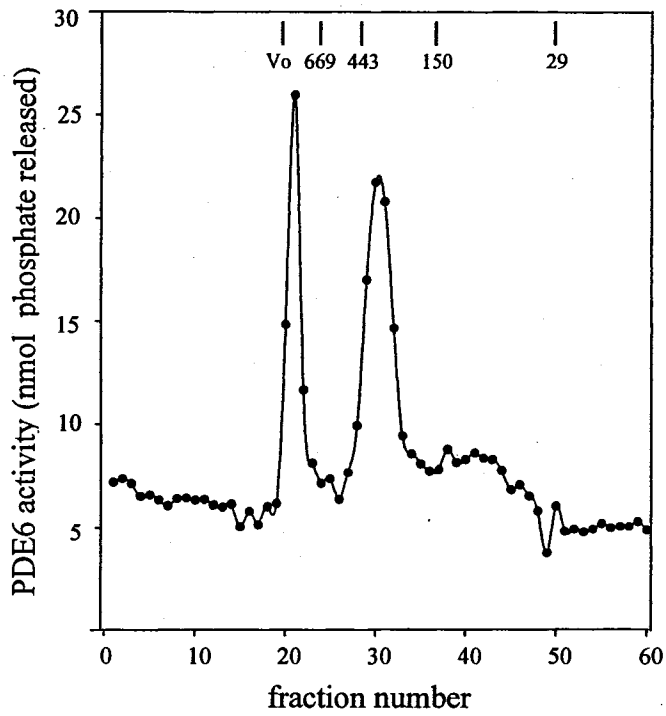

Figure 2.3. Size exclusion chromatography profile of bovine PDE6 protein complex solubilized using 10 mM CHAPS solution.
ROS membranes (200 μL of 100 μM Rho) were extracted with a solution containing 10 mM CHAPS. Following separation by size exclusion, fractions were analyzed for PDE6 activity. Two peaks of PDE6 activity are found, the first one eluting in the void volume of the column (Superdex 200), and assumed to be formed of detergent micelles, while the second peak has an apparent molecular mass of ~380kDa. Western blot analysis shows GARP2 and Tα co-eluting in the first PDE6 activity peak, but GARP2 or Tα not co-purifying with PDE6 in the second PDE6 activity peak (data not shown).

4. PDE6 protein complex released by binding to PrBP-δ.

PrBP-δ is an isoprenyl binding protein whose function in photoreceptors is unclear (Cook et al., 2001; Norton et al., 2005; Zhang et al., 2004). Nonetheless, because PrBP-δ can completely solubilize PDE6 from membranes (Florio et al., 1996; Norton et al., 2005), it can be used for studying PDE6 interacting complexes.

Purified recombinant bovine GST-PrBP-δ (30 PrBP-δ per PDE6) was used to solubilize the entire pool of PDE6 from ROS disk membranes. This extract was analyzed by size exclusion chromatography, and a single PDE6 activity peak was detected (see Fig. 2.4.). Examining the protein elution peak (from the OD_{280}) together with the PDE6 activity peak, it was determined that the apparent molecular weight of this peak was around 400 kDa. Analysis of the proteins present in this peak reveals the absence of GARP2. This result suggests that PrBP-δ dissociates GARP2 from PDE6 while it is solubilizing PDE6 from the ROS membranes. The higher molecular weight of the complex could be the result of the change in shape of the PDE6 complexed with PrBP-δ. It is not excluded that other proteins, not detected by us, also account for some or all of the increase in molecular size of the PDE6 complex solubilized by PrBP-δ.

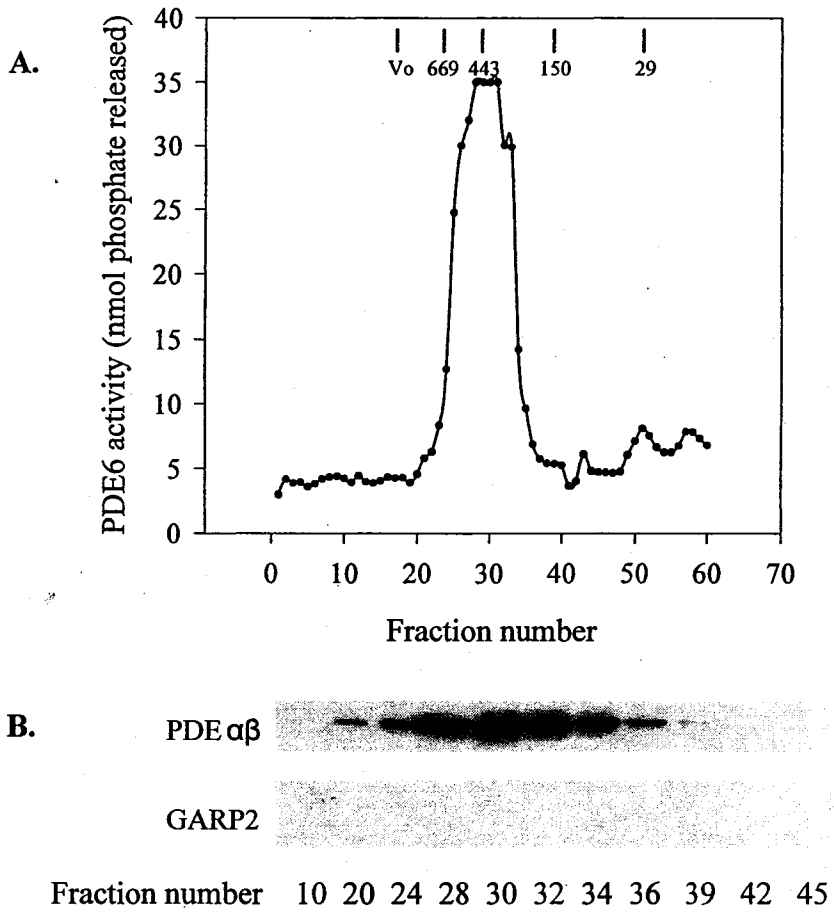

Figure 2.4. Size exclusion chromatography elution profile of PDE6 solubilized using PrBP-δ.
200 μL ROS of 100 μM [Rho] were treated with 30 PrBP-δ per PDE6 overnight. The supernatant was separated from the membrane pellet, and subjected to size exclusion chromatography. **A.** 300 μL fractions were collected and analyzed for PDE6 activity. One single peak of PDE6 activity was found, and was calculated to elute at an apparent molecular mass of ~400 kDa (±100 kDa). **B.** 10 μL of the peak fractions were analyzed on western blot with PDEαβ and GARP2 antibodies.

Further studies have been conducted to determine the apparent molecular masses of various preparations of PDE6 (see Table 2.1.).

Sample	Apparent molecular mass
Soluble PDE6 (isotonically extracted)	410 ± 30, n = 2
Trysinized soluble PDE6	363 ± 47, n = 3
Membrane-attached PDE6 (hypotonically extracted)	420 ± 10, n = 2
Trypsinized membrane-attached PDE6	315 ± 45, n = 4
PrBP-δ extracted PDE6	400, n = 1
CHAPS extracted PDE6	380, n = 1 (second peak)

Table 2.1. Size exclusion chromatography analysis of various PDE6 species.

To determine whether PDE6 shape might account for the increased apparent molecular weight, mildly trypsinized PDE6 was analyzed by size exclusion chromatography. Upon mild trypsinization, both GARP2 and Pγ are destroyed. By removing PDE6 associated proteins by trypsinization, we are able to obtain PDEαβ heterodimer free of other proteins. The apparent molecular mass of this protein was calculated and compared to the molecular mass obtained from size exclusion chromatography analysis. The apparent molecular weight decreases for both soluble PDE6 and membrane attached PDE6. Hypotonically extracted PDE6 has an apparent molecular mass slightly increased compared to detergent or PrBP-δ extracted PDE6. This suggests that hypotonic extraction is the method that preserves most protein interactions, while detergent and PrBP-δ disrupt some of those interactions.

Size exclusion chromatography cannot resolve the size of the protein complex from the shape of the molecule. An oblong protein with a lower molecular mass will elute at the same elution volume as a spherical protein with a higher molecular mass. In order to definitively determine the composition of a protein complex, additional analysis by SDS-PAGE followed by protein identification by mass spectrometry is necessary.

5. Disrupting the PDE6 multi-protein complex by varying salt concentrations.

Some of the proteins that interact with PDE6 probably have a low affinity of interaction which can be disrupted during the solubilization or purification process. PDE6 interacting proteins may come into contact with PDE6 only during certain steps of the activation pathway. For example, we hypothesize in chapter 4 that GARP2 does not interact with PDE6 throughout its activation cycle, but only at certain steps. Based on this assumption, it may be possible to selectively separate PDE6 from GARP2 using different solution conditions that disrupt their interaction. It has previously been shown that both PDE6 and GARP2 are membrane bound, and PDE6 cannot be released by an isotonic buffer solution (Kuhn, 1982). PDE6 remains membrane associated if ROS membranes are exposed to a hypotonic solution supplemented with 20 mM $MgCl_2$ (Kuhn, 1982). Activated transducin separates from PDE6 if differential ionic strength of the extraction buffer is used (Kuhn, 1982).

The experimental approach that we considered was to find a salt or $MgCl_2$ concentration that would selectively disrupt the interaction between PDE6 and GARP2 and release one protein from the ROS membranes. The solutions used were 5 mM Tris, pH. 7.5 and decreasing NaCl concentrations, from 150 mM to 10 mM. For the $MgCl_2$ solution, a 5 mM Tris, pH 7.5 buffer was supplemented with $MgCl_2$ concentrations ranging from 20 mM to 1 mM. We observed that as the salt concentration was decreased, PDE6 started to be released from ROS membranes at 25 mM NaCl, and was more than 90% solubilized at 10 mM NaCl (see Fig. 2.5.). GARP2 however, does not show a distinct pattern of solubilization from PDE6, and also started to be released from ROS membranes at 25 mM NaCl. For the $MgCl_2$ concentration series, GARP2 began to be

solubilized when $MgCl_2$ concentration reaches 5 mM. Traces of GARP2 are also released at 5 mM $MgCl_2$, but when $MgCl_2$ concentration is 2 mM, a large portion of PDE6, as well as GARP2, are solubilized. Further work is needed in order to optimize the separation conditions between PDE6 and GARP2. One approach is to induce various activation states of PDE6 using nucleotides, and to test the above-mentioned buffer compositions for each activation condition. The conclusion of this study is that under the experimental conditions tested, PDE6 and GARP2 are associated with relatively high affinity, and this affinity can not be differentially disrupted by the different ionic conditions used.

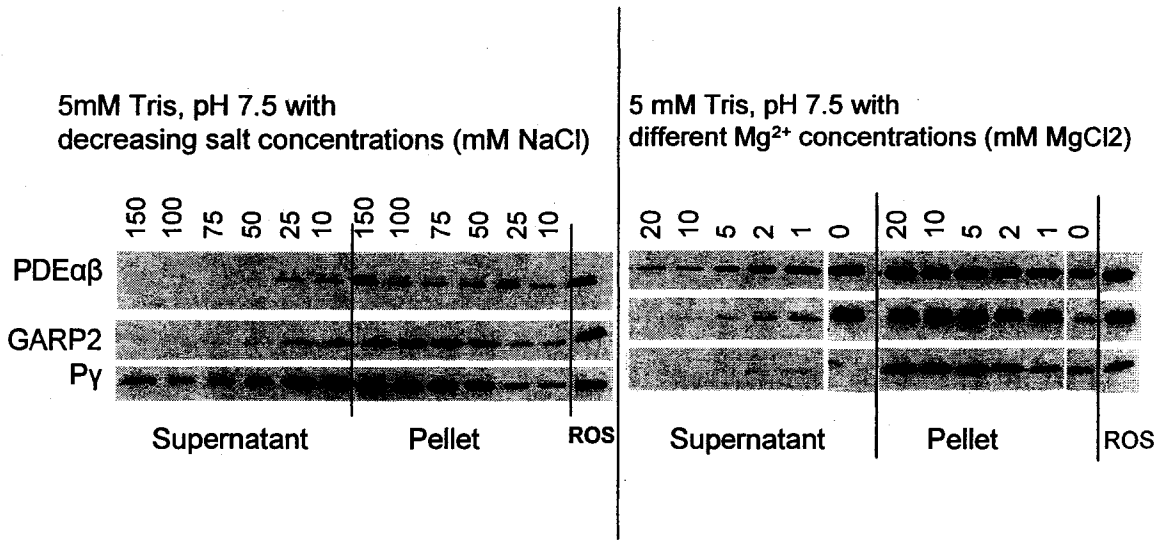

Figure 2.5. Attempts to selectively solubilize PDE6 from GARP2.
Bovine ROS membranes (100 μL of 50 μM Rho) were homogenized in the indicated buffers and the soluble fraction separated from the membranes by centrifugation for 5 minutes at 130,000 x g in an Airfuge. 10 μL of each supernatant and of the resuspended pellets were subjected to SDS-PAGE and western blotting. The blots were first probed for GARP2 using a full-length anti-GARP2 antibody, and were probed next for PDEαβ and Pγ with anti- catalytic subunit and C-terminus Pγ antibodies.

6. Purifying PDE6 from interacting proteins.

Based on the work described above, an improved purification scheme for PDE6 was developed.

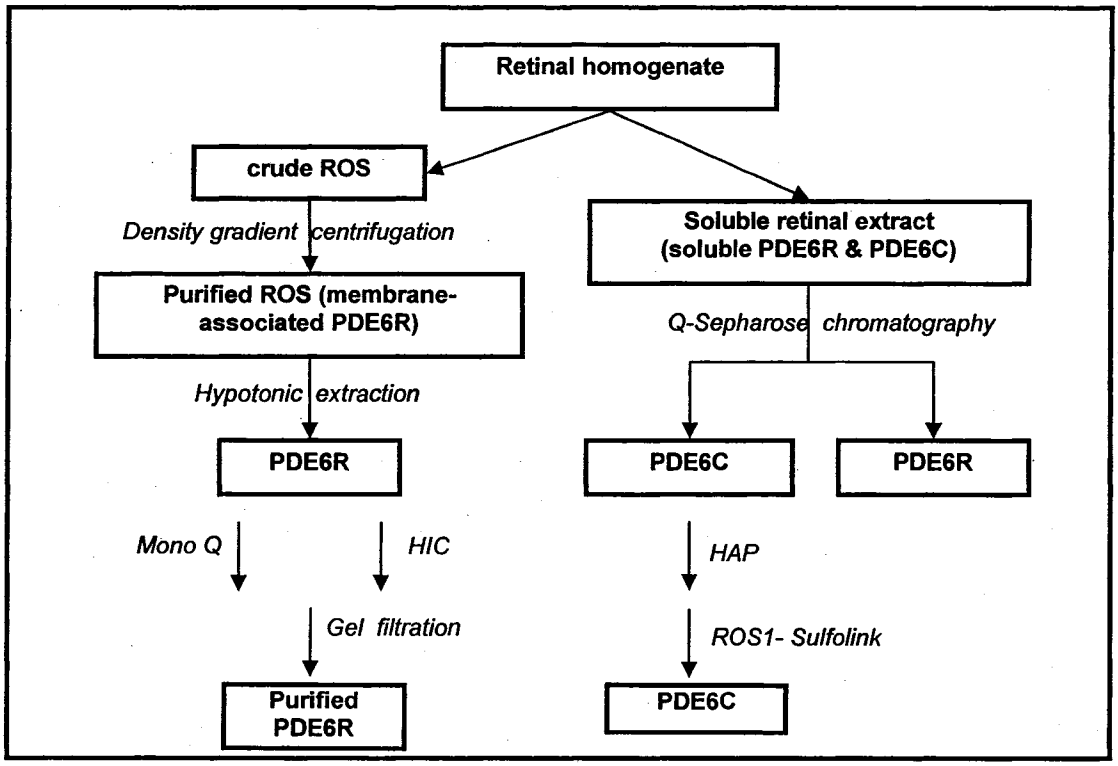

Figure 2.6. Purification scheme for PDE6.
Abbreviations are: MonoQ, anion exchange chromatography on a GE-Healthcare MonoQ column; HIC, hydrophobic interaction chromatography using butyl-Sepharose; HAP, hydroxyapatite chromatography; ROS1-Sulfolink, immunoafinity purification using monoclonal PDE6 specific antibody ROS1 (see Materials and Methods).

The retinal homogenate is the source for both ROS membranes, with their attached proteins, and soluble extract used for the purification of cone PDE6 and soluble rod PDE6. The procedures described below address only the purification to homogeneity of membrane-associated rod PDE6.

For the purification of ROS membranes, a discontinuous sucrose density gradient centrifugation step was required. Relying on the relative buoyancy of ROS, appropriate concentrations of sucrose will separate ROS free of other retinal cells and debris (McDowell, 1993); see Fig.2.6.). ROS obtained from 50 retinas contains 0.7-1 mg PDE6.

To extract PDE6 from ROS membranes, it is first necessary to remove the soluble proteins present in the ROS. By disrupting the plasma membrane of the ROS in a moderate ionic strength buffer in the dark, ROS membranes were separated from the soluble proteins by centrifugation (Baehr et al., 1979). The resulting ROS membranes contain integral membrane proteins (predominantly rhodopsin), along with peripheral membrane proteins (notably PDE6 and a reduced amount of transducin). Because transducin undergoes a light-dependent binding to photoactivated rhodopsin (Kuhn, 1982), the ROS membranes were exposed to light just before releasing PDE6 with a low ionic strength buffer.

The next step in the purification of PDE6 is anion-exchange chromatography by Mono Q. Anion-exchange chromatography of the PDE6 family has traditionally utilized the weak anion exchanger, diethylaminoethyl (DEAE), as the functional group (Gillespie and Beavo, 1988; Baehr et al., 1979). We find that greater reproducibility and better resolution are achieved for PDE6 isozymes when a strong anion exchanger (quaternary ammonium) is used. The purification of hypotonically extracted PDE6 on a Mono Q column eliminates most other proteins, PDE6 purifying with a greater than 50% purity. Membrane associated rod PDE6 elutes at ~400 mM NaCl concentration, with transducin subunits and GARP2 being the predominant impurity following this chromatographic step (Fig. 2.7).

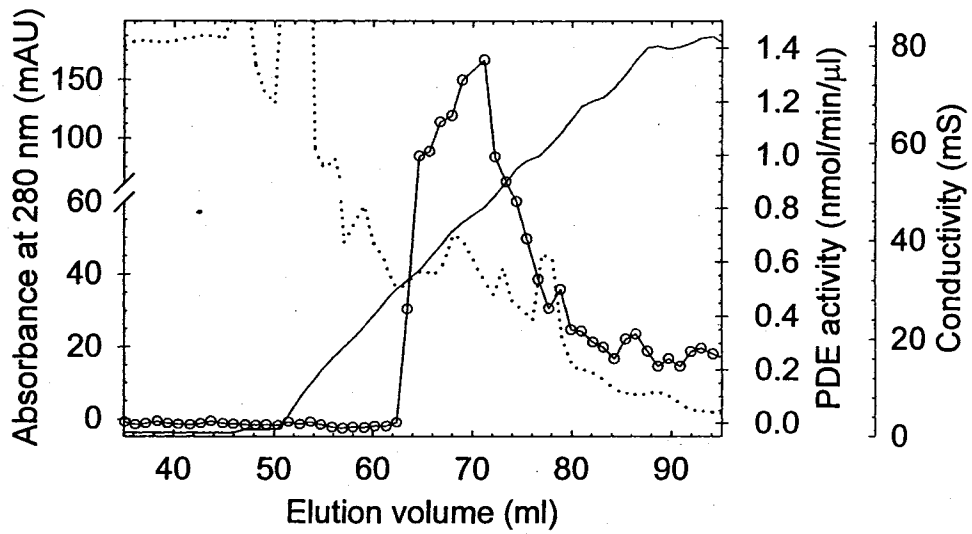

Figure 2.7. Mono Q chromatography of hypotonically extracted PDE6.
Forty-five milliliters of hypotonically extracted PDE6R was loaded onto a Mono Q column followed by 5 mL of MQ-A. PDE6R was eluted with a linear gradient from 0 to 100% MQ-B. For this particular experiment, the buffers were different from the standard procedure: MQ-A lacked NaCl, and MQ-B was 800 mM NaCl. Absorbance (dotted line) and conductivity (continuous line) were recorded, and PDE activity (—O—) was assayed for each fraction collected.

Gel filtration chromatography is the most suitable final step for PDE6 purification. It not only purifies PDE6 from other proteins based on size, but it also equilibrates the enzyme in a buffer more suitable for long-term storage (Fig. 2.8). The relative purity of PDE6 after this step is greater than 50%, with the major contaminants probably being proteolytic fragments of PDE6 and residual transducin subunits.

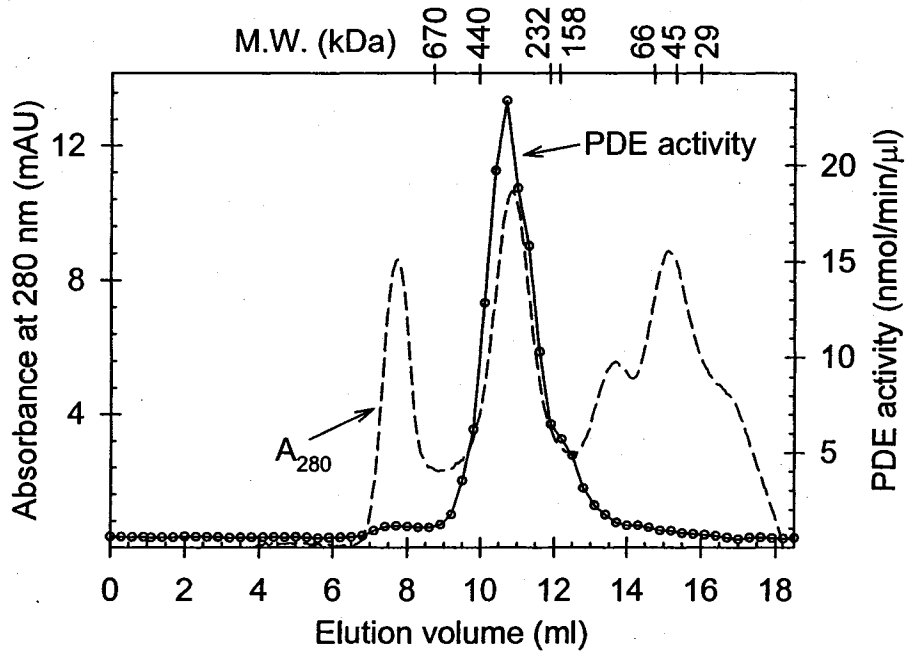

Figure 2.8. Gel filtration chromatography of PDE6 on Superdex 200.
A 0.5-mL sample of Mono Q-purified PDE6 was injected onto a Superdex 200 HR 10/30 column at 0.4-mL/min, and 0.3-mL fractions were collected. The 280-nm peak at 7.5 mL represents material in the void volume, with other impurities eluting at less than 100 kDa. The protein and PDE activity peak at 11.0 mL has an apparent mol mass of 300 kDa.

As a final step in PDE6 preparation is concentration by ultrafiltration. Centrifugal ultrafiltration serves three purposes: concentration of the PDE6 prior to storage, removal of low molecular weight impurities, and exchange of the purification buffer with the PDE storage buffer. In our experience, the Amicon/Millipore devices with the Ultracel PL membrane (Centricon and Centricon Plus-20) offer the best rate of concentration and highest recoveries for PDE6 isozymes.

The purity of PDE6 at different stages of purification is shown in Fig. 2.9.

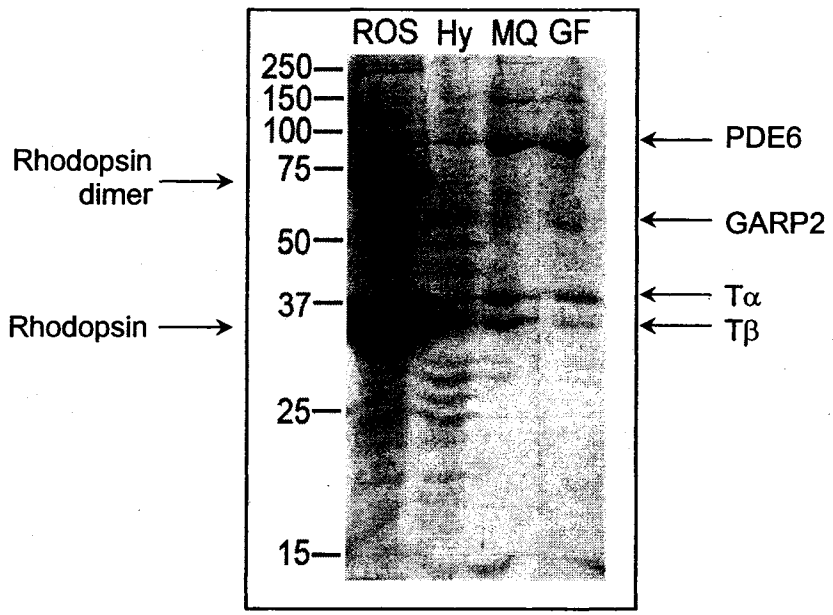

Figure 2.9. SDS-PAGE of membrane-associated PDE6 at various stages of purification.
Samples were applied to 12% acrylamide gels and electrophoresed and the gel stained with Coomassie Blue.

If ROS membranes contain about 1% PDE6 (Pugh, Jr. and Lamb, 1993), the hypotonic extraction step greatly increases PDE6 purification. The major protein of ROS membranes, rhodopsin (~70% of total protein), is not released by hypotonic extraction, being a transmembrane protein. Other transmembrane proteins, such as peripherin, or R9AP also are not released during the hypotonic extraction procedure. After hypotonic extraction, the major contaminating protein is transducin. In intact ROS, transducin is ten times more abundant than PDE6 (Pugh, Jr. and Lamb, 1993), and being a peripheral membrane protein is solubilized along with PDE6 under hypotonic conditions.

The next steps in PDE6 purification greatly reduce transducin contamination, as well as other proteins that co-solubilize with PDE6. After MonoQ chromatography, some

proteins with the similar chromatographic properties co-purify with PDE6. After this step, the sample contains ~30% PDE6. Gel filtration chromatography eliminates most proteins that are distinctly different in size from PDE6. In the end, the PDE6 sample is purified, with greater than 50% purity. One contaminant in the PDE6 sample is likely to be GARP2, which does not stain well with Coomassie Blue dye, therefore, it is difficult to accurately assess the degree of contamination in the final PDE6 sample.

7. Purifying the PDE6 catalytic dimer.

Because of the very high affinity with which Pγ binds to the catalytic dimer of PDE6 (Wensel and Stryer, 1988), the most effective way to prepare PDEαβ is to digest the Pγ subunits by limited proteolysis. Under controlled conditions, trypsin can effectively degrade the Pγ-subunits (and relieve their inhibition of the active site) without affecting the properties of the catalytic dimer of PDE6 (Hurley and Stryer, 1982; Catty and Deterre, 1991; Mou et al., 1999).

Although limited proteolysis with trypsin effectively activates PDE6 without adversely affecting the catalytic subunits, a large proteolytic fragment of Pγ consisting of the C-terminal half of the protein is generated (Fig. 2.10). This peptide has low affinity for binding to the active site of the enzyme (Artemyev and Hamm, 1992; Mou and Cote, 2001), but at high concentrations Pγ C-terminal peptides can act as competitive inhibitors of catalysis (Mou and Cote, 2001). MonoQ chromatography successfully removes the 5 kDa contaminating Pγ peptide from the PDEαβ dimer (see Fig. 2.10., last lane). For a complete removal of traces of the peptide, a gel filtration purification of the PDEαβ dimer is necessary.

Following this procedure for purifying PDEαβ (see Materials and Methods), a Pγ-free heterodimer sample is obtained, allowing an accurate study of the catalytic and regulatory properties of the catalytic dimer, and of the proteins that might interact with it. The time course of disappearance of the 11-kDa Pγ-subunit correlates with the appearance of an approx 5 kDa Pγ fragment (amino acids 45–87). The αand β catalytic subunits are not degraded by this treatment. Following purification of the trypsin-activated PDE6 on Mono Q (MQ-tPDE), most of the Pγ fragment is removed.

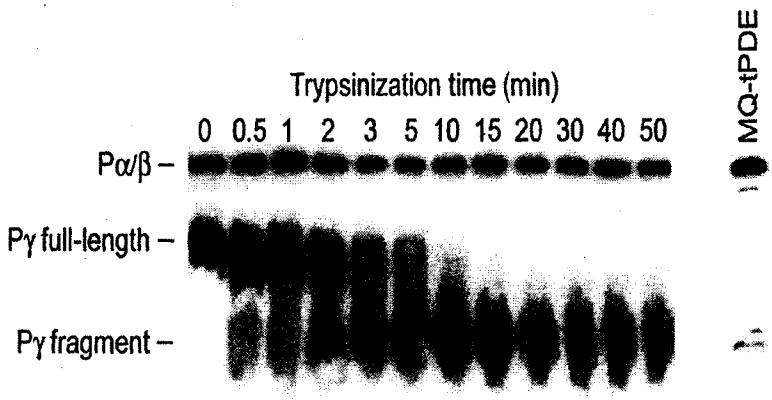

Figure 2.10. Proteolytic digestion of PDE6 holoenzyme preferentially destroys the Pγ-subunit.
PDE6 (50 nM) purified by Mono Q and gel filtration chromatography was incubated with 50 μg/mL of trypsin at 4°C. At the indicated times, samples were mixed with soybean trypsin inhibitor and run on SDS-PAGE. After transfer to a nitrocellulose membrane at 60 V for 1 h, the membrane was probed with a mixture of catalytic and inhibitory subunit antibodies.

Summary

This study analyzed proteins interacting with PDE6 during different stages of PDE6 purification or phototransduction activation. The various nucleotide conditions for activation of PDE6 were used to evaluate whether different proteins associate with PDE6

in an activation-dependent manner. It appeared that Tα is not co-solubilizing with PDE6 under light activation conditions, co-precipitating with PDE6 only for the dark-extracted sample. In the absence of an effective solubilization method that would not interfere with the affinities of the interacting proteins, different methods were assessed in their ability to solubilize and preserve protein interactions. The interacting proteins were evaluated in their relative affinities for PDE6 during the visual cycle. Also, the method chosen for isolation of the PDE6 protein complex may interfere with some protein-protein interactions, and can result in false positives for proteins of the same molecular weight as heavy or light chain of the immunoprecipitating antibody (e.g. GARP2). It is needed to further explore other methods for isolating the PDE6 protein complex, and for identification of the interacting proteins, such as the use of biotinylated antibodies to reduce heavy and light chain interference, analytical ultracentrifugation to determine the size and shape of the protein complex, and SDS-PAGE followed by mass spectrometry for identification of the unknown proteins. Based on the analysis of the efficiency of the solubilization methods used and on their ability to preserve or destroy protein interactions, a purification method for PDE6 was developed.

CHAPTER 3

THE GLUTAMIC ACID-RICH PROTEIN-2 (GARP2) IS A HIGH-AFFINITY ROD PHOTORECEPTOR PHOSPHODIESTERASE (PDE6) BINDING PROTEIN THAT MODULATES ITS CATALYTIC PROPERTIES[4]

Abstract

The glutamic acid-rich protein-2 (GARP2)[5] is a splice variant of the β-subunit of the cGMP-gated ion channel of rod photoreceptors. GARP2 is believed to interact with several membrane-associated phototransduction proteins in rod photoreceptors. In this paper, we first demonstrate that GARP2 is a high-affinity PDE6 binding protein, and that PDE6 co-purifies with GARP2 during several stages of chromatographic purification. We find that hydrophobic interaction chromatography succeeds in quantitatively separating

[4] This paper was published in the Journal of Biological Chemistry: Pentia, D.C., Hosier, S., Cote, R.H. 2006 Mar 3;281(9):5500-5

[5] The abbreviations used are: GARP2, glutamic acid-rich protein 2; PDE6, photoreceptor phosphodiesterase; Pγ, inhibitory 10 kDa γ subunit of PDE6; PrBP/δ, 17 kDa prenyl-binding protein; GST, glutathione S-transferase; GTPγS, guanosine 5'-3-O-(thio)-triphosphate; ROS, rod outer segment; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; HIC, hydrophobic interaction chromatography.

GARP2 from the PDE6 holoenzyme. Furthermore, the 17 kDa prenyl binding protein abundant in retinal cells selectively releases PDE6—but not GARP2—from rod outer segment membranes, demonstrating the specificity of the interaction between GARP2 and PDE6. Purified GARP2 is able to suppress 80% of the basal activity of the non-activated, membrane-bound PDE6 holoenzyme at concentrations equivalent to its endogenous concentration in rod outer segment membranes. However, GARP2 is unable to reverse transducin activation of PDE6 (in contrast to a previous study), nor does it significantly alter catalysis of the fully activated PDE6 catalytic dimer. The high binding affinity of GARP2 for PDE6 and its ability to regulate PDE6 activity in its dark-adapted state suggest a novel role for GARP2 as a regulator of spontaneous activation of rod PDE6, thereby serving to lower rod photoreceptor "dark noise" and allowing these sensory cells to operate at the single photon detection limit.

Introduction

The visual transduction pathway in vertebrate photoreceptors is remarkable in many respects, including single photon detection capability (in rod photoreceptors), photoresponse kinetics on the millisecond time scale, and the ability to adapt to background illumination levels ranging from very dim illuminance levels (scotopic vision in rods) to bright sunlight (photopic vision in cones) (Rodieck, 1998). The very first steps in vision occur in the photoreceptor outer segment when photoisomerized rhodopsin activates the heterotrimeric G-protein, transducin, which proceeds to bind to and displace the inhibitory γ subunit ($P\gamma$) of the photoreceptor phosphodiesterase (PDE6). Activated PDE6 rapidly lowers the cGMP concentration, resulting in closure of cGMP-gated channels in the plasma membrane and cell hyperpolarization (Burns and Baylor, 2001; Arshavsky et al., 2002; Zhang and Cote, 2005). Several feedback mechanisms operate to actively terminate the photoresponse and restore the dark-adapted state, of which regulation of the lifetime of activated transducin is considered rate-limiting (Arshavsky et al., 2002; Burns and Baylor, 2001). Re-binding of $P\gamma$ to the PDE6 catalytic subunits following transducin deactivation returns PDE6 to its nonactivated state and allows cGMP levels to return to their dark-adapted levels.

Electrophysiological evidence supports the hypothesis that factors in addition to transducin deactivation are involved in regulating the lifetime of light-activated PDE6 during light adaptation of rod photoreceptors (Calvert et al., 2002; Krispel et al., 2003).

Several potential feedback mechanisms for modulating activated PDE6 have been proposed (Erickson et al., 1992; Korschen et al., 1999; Hayashi et al., 2000), but have not been explored in sufficient detail to validate their relevance to the phototransduction pathway.

The catalytic activity of PDE6 in its dark-adapted state also must be tightly controlled to prevent spontaneous activation of PDE6 that would consume metabolic energy unnecessarily and impair the ability of rod cells to reliably detect very dim flashes of light. Physiological measurements of "dark noise" reveal a component that represents spontaneous activation of PDE6, and which is much greater in magnitude in cones than in rods (Rieke and Baylor, 1996; Rieke and Baylor, 2000; Holcman and Korenbrot, 2005). Subtle differences in the highly homologous rod and cone isoforms of PDE6 might account for the different "dark noise" in rods and cones, although this is not evident from biochemical comparisons of purified rod and cone PDE6 (Baehr et al., 1979; Gillespie and Beavo, 1988; Mou et al., 1999; Huang et al., 2004). An alternative possibility is that a rod- or cone-specific PDE6 binding protein suppresses the spontaneous activation of PDE6 by enhancing the affinity of Pγ at the PDE6 catalytic site.

One candidate protein that might serve to regulate PDE6 in both its nonactivated and activated states is the glutamic acid-rich protein-2 (GARP2), a protein that exists in rod outer segments but is absent in cones (Colville and Molday, 1996; Korschen et al., 1999). GARP2 is a product of alternative splicing of the β-subunit of the rod cGMP-gated ion channel (CNGB1) and contains a unique 8-amino acid C-terminal extension (Colville and Molday, 1996; Korschen et al., 1999). This 32 kDa protein is unusual in that it has a high

content of proline and glutamate residues (Sugimoto et al., 1991; Colville and Molday, 1996; Korschen et al., 1995).

The functions served by GARP2 in rod outer segments are unknown. Potential binding partners for GARP2 include proteins involved in phototransduction and disk membrane structural integrity (Korschen et al., 1999; Poetsch et al., 2001) but the physiological significance of these interactions is unclear. In one previous study, it was reported that addition of GARP2 to preparations of PDE6 reversed its activation by transducin, whereas GARP2 had no effect on the nonactivated PDE6 holoenzyme or on the catalytic dimer of PDE6 lacking bound Pγ (Korschen et al., 1999). It was proposed that GARP2 down-regulation of PDE6 activation in the vicinity of the plasma membrane might conserve metabolic energy during daylight when rod function is saturated.

In this paper we examine the interaction of GARP2 with PDE6, and characterize the effect of GARP2 on PDE6 function. We show that GARP2 binds PDE6 with high affinity, and co-purifies with the enzyme through several stages of purification. We have been unable to confirm the previously reported inhibitory effect of GARP2 on transducin-activated PDE6 (Korschen et al., 1999). Instead, we observe that purified, native GARP2 has a strong effect in suppressing the basal activity of PDE6 in its nonactivated state. The implications of GARP2 modulation of basal PDE6 activity in dark-adapted rods are discussed.

Materials and Methods

Materials—Bovine retinas were purchased from W. L. Lawson, Inc. Chromatography supplies were purchased from GE Healthcare and Pierce. Supplies for immunoblotting were purchased from Schleicher & Schuell, Pierce, and Bio-Rad. Chemicals were obtained from Sigma. The bovine recombinant GST-PrBP/δ fusion protein was a kind gift of Dr. Joe Beavo (Univ. of Washington). Rabbit polyclonal anti-GARP2 antibody to the unique C-terminal sequence of GARP2 (Colville and Molday, 1996) was obtained from Affinity Bioreagents (catalog #PA1-728). Chicken and rabbit polyclonal anti-GARP antibodies to bovine sequences common to GARP1, GARP2, and the rod β-subunit CNGB1 were kind gifts of Dr. Steven Pittler (Univ. Alabama) and Dr. Benjamin Kaupp (Inst. Biol. Inform., Jülich). Affinity-purified anti-peptide rabbit polyclonal antibodies directed to the PDE6 GAFb domain (termed NC) and to the C-terminus of the Pγ subunit of PDE6 (CT-9710) were produced in our laboratory. The ROS1 monoclonal antibody used for immunoprecipitations (Hurwitz et al., 1984) was a gift of Dr. Richard Hurwitz (Baylor College of Medicine).

ROS membrane isolation and purification—ROS membranes from bovine retina were prepared as described previously (Pentia et al., 2005). Briefly, ROS were isolated from frozen bovine retinas on a discontinuous sucrose gradient. ROS membranes were homogenized in an isotonic buffer (10 mM Tris, pH 7.5, 60 mM KCl, 40 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 0.3 mM PMSF) using a glass, handheld homogenizer. The soluble proteins were separated from membranes by centrifugation.

Native GARP2 purification—GARP2 was isolated from ROS membranes and purified to homogeneity as follows. First, ROS membranes were homogenized in a hypotonic buffer (5 mM Tris, pH 7.5, 0.2 mM $MgCl_2$, 1 mM DTT). The soluble proteins were separated from membranes by centrifugation at 100,000 *g* for 45 min. The hypotonic extraction was repeated three times. The pooled hypotonic extract was then adjusted to 500 mM ammonium sulfate and applied to a 15 ml Butyl-Sepharose column. The column was washed of unbound proteins using two column volumes of 500 mM ammonium sulfate in 5 mM Tris, pH 7.5, and bound proteins were eluted by a step gradient: 400 mM ammonium sulfate, 150 mM ammonium sulfate, and no ammonium sulfate in a solution containing 5 mM Tris, pH 7.5, 1 mM DTT. The GARP2-containing fractions were pooled, adjusted to 500 mM ammonium sulfate, and re-chromatographed on Butyl-Sepharose. To concentrate and further purify GARP2 from other contaminating proteins, anion exchange chromatography on Mono Q was used exactly as described for PDE6 purification.

In some instances, the GARP2-containing fractions from the first Butyl-Sepharose column were chromatographed on a Mono Q column prior to a final purification using a reversed phase HPLC column (Vydac 214TP54) with a gradient of 0 to 100% acetonitrile containing 0.1% trifluoroacetic acid. Under these conditions, GARP2 eluted at 48% acetonitrile, Pγ was found at 43% acetonitrile, and PDE6 catalytic subunits were undetectable by immunoblot analysis. HPLC-purified GARP2 behaved identically to Butyl-Sepharose purified GARP2 in its effects on PDE6 catalysis.

PDE6 purification—Purified PDE6 was prepared as described elsewhere (Pentia et al., 2005). Briefly, a hypotonic extract of purified ROS membranes was loaded onto a

MonoQ column. The proteins were eluted using a linear gradient from 100 mM NaCl to 1 M NaCl in 5 mM Tris, pH 7.5. The PDE6 peak was collected, concentrated, and further purified on a Superdex 200 gel filtration column using the following buffer: 5 mM Tris, pH 7.5, 300 mM NaCl, 1 mM DTT and 3 mM PMSF. The gel filtration column was calibrated using the following: thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (43 kDa), ovalbumin (29 kDa), Blue Dextran for the void volume and adenosine triphosphate for the included volume.

Immunoprecipitation of PDE6 with the monoclonal antibody ROS1—The ROS1 antibody to PDE6 (Hurwitz et al., 1984) coupled to Sulfolink beads (Pierce) was used for immunoprecipitation of PDE6 and its binding partners. Hypotonic extracts from bovine ROS or MonoQ-purified PDE6 (containing 5 to 10 pmol of PDE6) were incubated for 2 hours at 4°C with 20 µl pre-washed ROS1 beads in a total volume of 100 µl. Samples were centrifuged to separate bound from unbound proteins, and beads were washed extensively before proteins were eluted in Laemmli sample buffer. As a control for non-specific binding of GARP2 to the beads, purified GARP2 was also tested with the ROS1-Sulfolink beads. Portions of the starting material, bound proteins and unbound proteins were subjected to SDS-PAGE, followed by Western blotting for PDE6 (NC antibody) and GARP (chicken anti-GARP antibody).

PrBP/δ expression and purification—Recombinant bovine PrBP/δ was expressed in the *Escherichia coli* strain BL21 (Norton et al., 2005). Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactopyranoside to log phase cultures. Bacterial cells were incubated for 1h at 37°C, lysed by sonication, and soluble proteins were recovered following centrifugation. GST-PrBP/δ was purified on a glutathione-agarose

column. GST-PrBP/δ concentration was determined by a colorimetric protein assay (Smith et al., 1985).

Protein quantification—The amount of rhodopsin in dark-adapted ROS membranes was determined by difference spectroscopy (Bownds et al., 1971). The PDE6 concentration was routinely determined by measurements of trypsin-activated PDE6 maximum activity [V_{max}; (Cote, 2000)] and knowledge of the turnover number (k_{cat} = 5600 cGMP/s/PDE6; ref. (Mou and Cote, 2001)]: [PDE6] = V_{max}/k_{cat}. Independent determinations of the ratio of rhodopsin to PDE6 in purified bovine ROS gave a value of 310 ± 20 rhodopsins per PDE6 (n = 5), very similar to the values for amphibian ROS of 270 (Dumke et al., 1994) to 330 (Cote and Brunnock, 1993) rhodopsins per PDE.

The amount of purified GARP2 was routinely estimated by immunoblot analysis. Samples of purified GARP2 and known amounts of ROS membranes were resolved on SDS-PAGE and immunoblotted. GARP2 was detected using a GARP2-specific antibody. The intensities of GARP2 immunoreactive bands were determined using Quantiscan (Biosoft), and compared to GARP2 immunoreactivity in ROS membranes containing known amounts of rhodopsin and PDE6.

SDS-PAGE and Western blotting—SDS-PAGE was performed by the method of Laemmli (Laemmli, 1970) in 10%, 12% or 15% acrylamide gels. The immunoblotting procedure followed the protocols in Gallagher (Gallagher, 1998). Note that GARP2 typically migrates at ~60 kDa (roughly 2-fold higher than predicted based on its amino acid sequence) and shows size heterogeneity, in accord with previous observations (Korschen et al., 1995; Korschen et al., 1999). This anomalous behavior was recently

explained as being due to GARP2 existing in solution as a natively unfolded protein (Batra-Safferling et al., 2006).

Results and Discussion

GARP2 is a high-affinity PDE6 binding protein—Because there is uncertainty about the proteins with which GARP2 interacts in rod photoreceptors (Korschen et al., 1999; Poetsch et al., 2001), we first re-examined whether PDE6 interacts in a specific manner with GARP2. Following homogenization of purified ROS from bovine retina and removal of soluble proteins with isotonic washes, PDE6 and GARP2 both remain associated with ROS disk membranes (Fig. 3.1. A). Release of PDE6 from ROS membranes by exposure to a hypotonic buffer also causes the release of almost all detectible GARP2 (Fig. 3.1. A). Subsequent purification of PDE6 by anion-exchange chromatography on a MonoQ column results in co-elution of PDE6 and GARP2 at 400 mM NaCl. Gel filtration chromatography of the Mono Q-purified GARP2-PDE6 also failed to separate GARP2 from PDE6 (Fig. 3.1. A). The results in Fig. 3.1. A conclusively demonstrate that PDE6 and GARP2 are both associated with ROS membranes, are co-eluted by exposure to a hypotonic buffer, and co-purify by two different chromatographic procedures.

To directly show that GARP2 is associated with PDE6, we immunoprecipitated PDE6 with the ROS1 antibody (Hurwitz et al., 1984) coupled to Sulfolink beads. Fig. 1B shows that unpurified PDE6 obtained from hypotonic extraction of ROS membranes is pulled down in a complex with GARP2, and that very little GARP2 remains unbound under these conditions. MonoQ-purified PDE6 is also immunoprecipitated in tight

association with GARP2, whereas purified GARP2 fails to bind to the ROS1 antibody in the absence of PDE6 (Fig. 3.1. B).

To estimate whether a significant amount of the PDE6 exists free of bound GARP2, we performed gel filtration chromatography on proteins solubilized from dark-adapted ROS membranes with a hypotonic buffer. A single peak of PDE6 hydrolytic activity (Fig. 3.2. A) and immunoreactivity (Fig. 3.2. B) was observed at an apparent molecular weight of 330 kDa. [The higher than predicted molecular weight for the PDE6-GARP2 complex by gel filtration chromatography may be due to its asymmetric shape (Gillespie and Beavo, 1988).] Qualitatively, the observed ratio of PDE6 and GARP2 immunoreactivity did not vary in the fractions containing PDE6, indicating that there is not a large fraction of the total PDE6 that exists free of bound GARP2. Further, only small amounts of GARP2 immunoreactivity could be detected at an apparent molecular weight of ~30-60 kDa (Fig. 3.2. B). This result indicates that PDE6 is tightly associated with GARP2 and that there is no evidence for a significant amount of unbound GARP2 or PDE6.

To assess whether GARP2 binding to PDE6 might be an artifact of the initial hypotonic extraction of ROS membrane proteins, we also solubilized PDE6 and GARP2 from dark-adapted ROS membranes with 1% Triton X-100. After removing the ROS membranes by centrifugation and immunoprecipitation of the detergent-solubilized PDE6 with the ROS1 antibody, we detected both PDE6 and GARP2 in the immunoprecipitates; control samples with beads lacking the ROS1 antibody failed to pull down either protein (data not shown).

Together, these results demonstrate that most of the GARP2 in ROS co-purifies with PDE6 through several stages of purification. The fact that GARP2 remains bound to

PDE6 after repeated washing of the ROS1 immune complex (Fig. 3.1. B) and that little unbound GARP2 is observed during gel filtration chromatography (Fig. 3.2.) demonstrates that GARP2 binds PDE6 with high affinity.

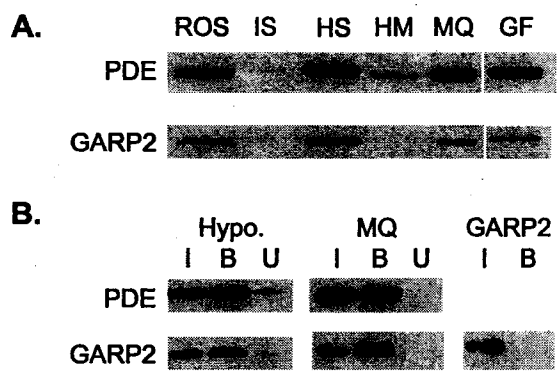

Figure 3.1. GARP2 is a high affinity PDE6 binding protein. A: Immunoblot of PDE6 samples during purification. Purified ROS membranes (ROS), hypotonic extract of ROS membranes (HS), MonoQ purified PDE6 (MQ) and gel filtration purified PDE6 (GF) lanes were each loaded with 1 pmol of PDE6. For the isotonic supernatant (IS), a volume equivalent to the original ROS homogenate was loaded; for the ROS membranes following hypotonic extraction (HM), an amount of rhodopsin equivalent to the ROS sample was loaded. The blots were probed with the PDE6 NC antibody and the GARP2-specific antibody.
B. Co-immunoprecipitation of PDE6 with GARP2. Hypotonically extracted PDE6 (Hypo.), MonoQ-purified PDE6 (MQ) or purified GARP2 were incubated with ROS1 antibody attached to Sulfolink beads (see Experimental Procedures). Samples representing 10% of the total PDE6 sample (input, I), 50% of bound proteins (B), and 10% of the unbound proteins (U) were run on 15% SDS-PAGE, and immunoblots were examined with antibodies specific for PDE6 and GARP. As a control for non-specific binding of GARP2 to the beads, immunoprecipitation was also carried out on PDE6-free purified GARP2, and samples representing 10% of the input and 50% of the pellet were analyzed. *(this result was obtained by Suzanne Hosier)*

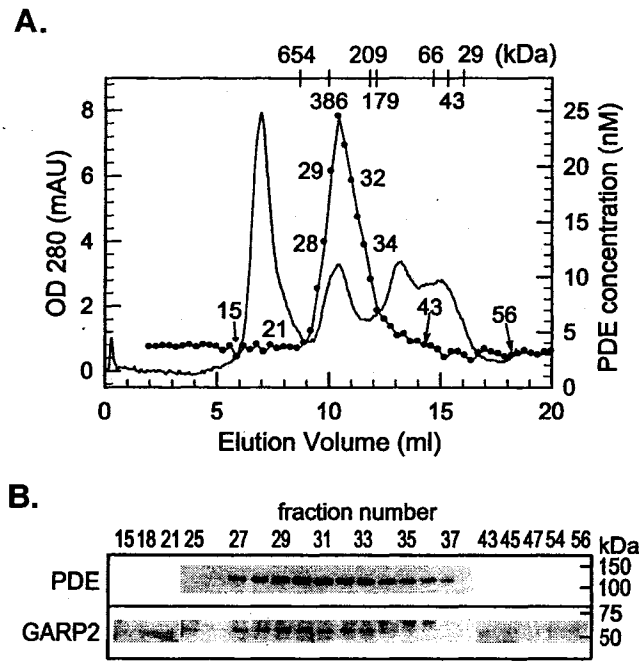

Figure 3.2. GARP2 and PDE6 co-elute upon size exclusion chromatography. A: A hypotonic extract of ROS membrane proteins (200 nM PDE) was loaded on a Superdex 200 column. The protein absorbance profile (continuous line) and the PDE6 activity (filled circles) are shown. The top x-axis shows the molecular mass of protein standards run on the column. Numbers adjacent to data points refer to fractions used for immunoblot analysis.
B. Immunoblots of a fixed volume of the indicated column fractions were probed with PDE6 and GARP2-specific antibodies.

GARP2 content in rod photoreceptors—If GARP2 is to regulate PDE6 activity during phototransduction, it would need to be present in ROS in molar equivalence to PDE6. A previous study by Kaupp and colleagues has suggested that GARP2 is actually ~3-fold more abundant than PDE6 [1 GARP2 per 100 rhodopsins (Korschen et al., 1999)]. Another GARP2 interacting protein, peripherin, is believed to bind only 10% of the total GARP2 in ROS (Poetsch et al., 2001). Our observation that practically all

GARP2 in bovine ROS co-purifies with PDE6 (Fig. 3.1. and Fig. 3.2.) suggests that GARP2 is not significantly more abundant than PDE6 in ROS.

To directly address the question of the GARP2 content in ROS, we purified native GARP2 from bovine ROS as described below, and compared the immunoreactivity of known amounts of GARP2 to that of intact ROS whose rhodopsin and PDE6 concentration were measured. The precision of our measurements were hampered by uncertainties in the concentration of purified GARP2 used for quantitative immunoblots, because the poor staining of GARP2 by Coomassie and other protein stains limited our ability to assess its purity on SDS-PAGE (see next section). Taking this into consideration, we estimate that there are 1-2 GARP2 molecules per PDE6 holoenzyme in bovine ROS (data not shown). This value agrees well with two other reports (Korschen et al., 1999; Batra-Safferling et al., 2006), and indicates that GARP2 is present in rod photoreceptors in sufficient amounts to bind all of the PDE6.

Purification of native GARP2, free of contamination with PDE6—In order to study the effects of GARP2 on PDE6 catalytic activity, we needed to purify GARP2 free of PDE6 subunits. We discovered that the association of GARP2 with PDE6 could be disrupted using high concentrations of ammonium sulfate. This permitted separation of the two proteins by hydrophobic interaction chromatography (HIC).

A hypotonic extract containing PDE6 and GARP2 was mixed with 500 mM ammonium sulfate, and the sample was applied to a Butyl-Sepharose column. A decreasing, discontinuous ammonium sulfate gradient permitted the separation of PDE6 (at higher ammonium sulfate concentrations) from the GARP2 (which eluted only when ammonium sulfate was omitted from the buffer (Fig. 3.3. A). Examination of the PDE6

peak by immunoblot analysis revealed no detectible GARP2 (Fig. 3.3. B, "HIC-PDE"). The disruption of GARP2 binding to PDE6 by ammonium sulfate suggests that hydrophobic domains in GARP2 (which contains 27% hydrophobic amino acids) may be important in promoting its binding to PDE6 catalytic subunits.

The GARP2 peak eluting from the Butyl-Sepharose column in the absence of ammonium sulfate still contained traces of the inhibitory Pγ subunit immunoreactivity at ~12 kDa, as well as a faint band of GARP1 immunoreactivity at ~130 kDa (Fig. 3.3. B, "HIC1"). By exposing the partially purified GARP2 sample to 500 mM ammonium sulfate and running the sample on Butyl Sepharose again, most of the residual Pγ was removed from the GARP2 (Fig. 3.3. B, "HIC2"). Alternatively, the GARP2-enriched fractions from the Butyl-Sepharose could be completely separated from PDE6 subunits by reversed phase HPLC.

On Coomassie-stained gels of purified GARP2, greater than 50% of the total staining is observed at ~60 kDa, corresponding to GARP2 (Fig. 3.3. C). No detectable protein is observed at molecular weights corresponding to PDE6 catalytic or inhibitory subunits. The GARP2 purity is likely to be much higher, since this glutamate-rich protein binds Coomassie protein stain very poorly relative to other proteins. It is therefore unlikely that the effects of GARP2 on PDE6 activity reported below can be ascribed to a contaminating protein in our purified GARP2.

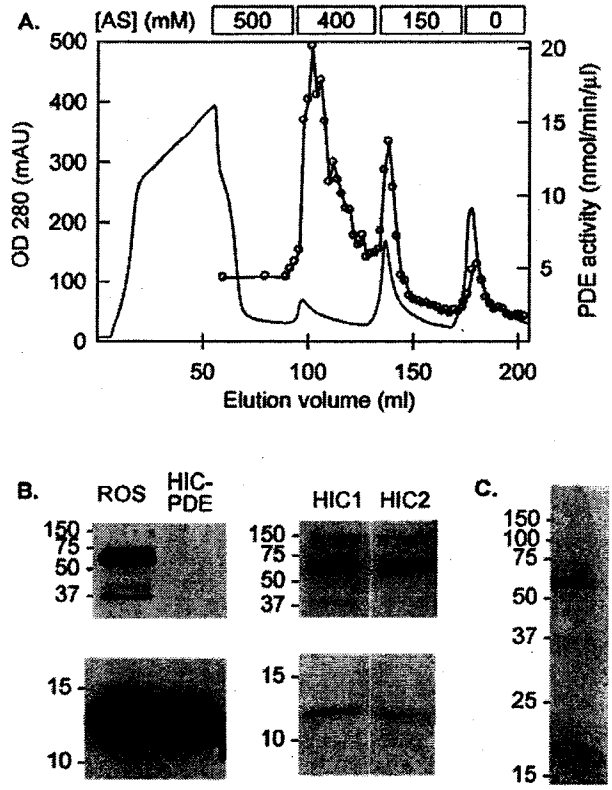

Figure 3.3. Separation of GARP2 from PDE6 by Butyl Sepharose chromatography. **A:** A hypotonic extract (containing 2.2 nmol PDE6) was adjusted to 500 mM ammonium sulfate concentration before being loaded on a 15 ml Butyl-Sepharose column. The ammonium sulfate concentration was lowered in steps as indicated at the top of the graph, and verified by conductivity measurements. The protein absorbance (continuous line) and PDE6 activity profile (filled circles) were determined. **B:** Immunoblots showing the starting material (ROS membranes; 0.5 pmol PDE), the PDE6 peak from Butyl-Sepharose chromatography (0.5 pmol PDE6), and equivalent volumes of GARP2 following one or two purifications of the GARP2 peak eluting at zero ammonium sulfate concentration. Immunoblots were probed with the rabbit anti-GARP antibody (upper panels), and the anti-Pγ subunit antibody (CT-9710; lower panels). C: HPLC-purified GARP2 (5 µg protein) was loaded on a 10% polyacrylamide gel, and the separated proteins stained with Coomassie dye. The position and size (in kDa) of protein standards are indicated.

The 17 kDa prenyl binding protein (PrBP/δ) releases PDE6, but not GARP2, from ROS membranes—The 17 kDa prenyl-binding protein (PrBP/δ)—originally described as the δ subunit of PDE6 (Gillespie et al., 1989)—is able to solubilize membrane-associated

rod PDE6 *in vitro* (Florio et al., 1996; Norton et al., 2005) by binding to the hydrophobic prenyl groups attached to the C-termini of the PDE6 catalytic subunits (Cook et al., 2000). Because hydrophobic interactions may stabilize GARP2-PDE6 interactions (see above), we wondered whether PrBP/δ binding to PDE6 would solubilize the enzyme as a complex with GARP2 or, alternatively, compete with GARP2 for binding to a hydrophobic region on PDE6. Fig. 3.4 shows an experiment in which increasing amounts of PrBP/δ are added to ROS membranes (containing bound PDE6 and GARP2), and the solubilization of PDE6 monitored by centrifugal separation of bound and soluble PDE6. While PDE6 catalytic and Pγ subunits are released from ROS membranes by PrBP/δ in a concentration-dependent manner, GARP2 remains completely associated with ROS membranes (Fig. 3.4).

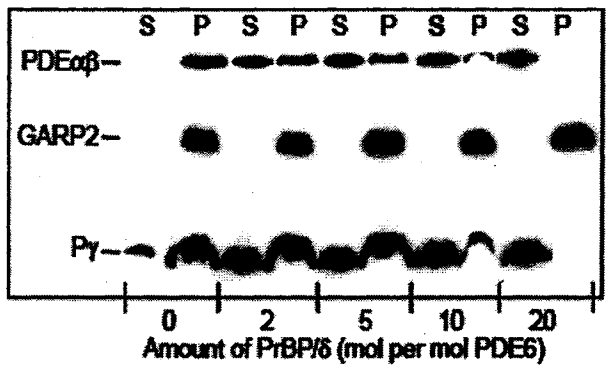

Figure 3.4. PrBP/δ selectively solubilizes PDE6 from ROS membranes while GARP2 remains on the membranes, free of PDE6 subunits. Purified ROS membranes were incubated overnight at 4°C with indicated amounts of GST-PrBP/δ (relative to the PDE6 subunit concentration). Membranes were centrifuged and soluble (S) and membrane fractions (P) were analyzed by immunoblotting using a GARP2-specific antibody, the PDE6 NC antibody, and the Pγ CT-9710 antibody.

This effect of PrBP/δ to disrupt GARP2-PDE6 interactions is not restricted to PDE6 associated with ROS membranes. If PDE6 and GARP2 are first released from ROS membranes by hypotonic extraction and then PrBP/δ is added, some of the GARP2 that

normally co-migrates with PDE6 at ~300 kDa during gel filtration chromatography is now observed eluting at an apparent molecular weight of ~60 kDa (data not shown). The disruption of GARP2-PDE6 interactions by PrBP/δ suggests that one site of interaction may be at the hydrophobic isoprenyl groups at the C-termini of PDE6 catalytic subunits. It is possible that this same binding interface may be disrupted during hydrophobic interaction chromatography.

Purified, native GARP2 suppresses basal PDE6 catalytic activity, but does not inhibit activated PDE6—It was previously reported that GARP2 potently inhibited PDE6 hydrolytic activity when purified PDE6 was activated by transducin in solution; in contrast, trypsin-activated PDE6 (lacking Pγ) or nonactivated enzyme ($\alpha\beta\gamma\gamma$) were not greatly affected by GARP2 (Korschen et al., 1999). However, Korschen et al. used a recombinant GARP2 fusion protein for these experiments, and unpublished results from the same group have called into question the ability of native GARP2 to inhibit transducin-activated PDE6 (Kaupp and Seifert, 2002). Furthermore, transducin activates PDE6 more effectively when both proteins are associated with the disk membrane compared to activation in solution (Fung and Nash, 1983; Bruckert et al., 1994). Therefore, we chose to examine whether native, purified GARP2 exerted an effect on nonactivated or activated PDE6 under more physiological conditions in which PDE6 remains associated with the disk membrane.

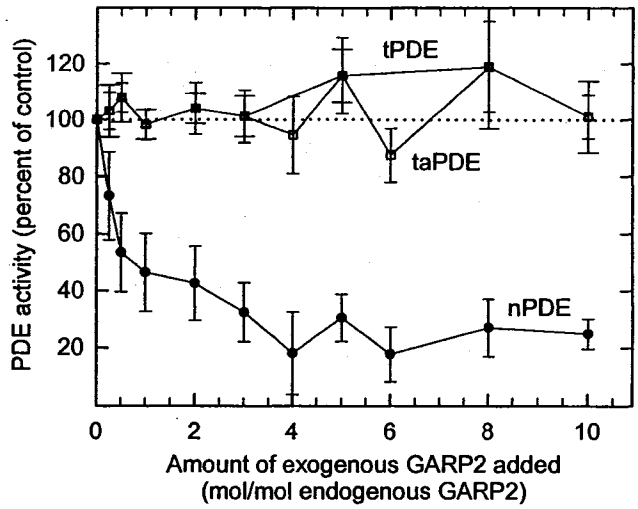

Figure 3.5. GARP2 suppresses nonactivated PDE6 activity but is ineffective with activated PDE6. Bovine ROS homogenates (10 nM PDE6) were either trypsin-activated (tPDE), transducin-activated by addition of 10 μM GTPγS (taPDE), or maintained in a non-activated state (nPDE), as described in Experimental Procedures. Purified GARP2 was added to each sample in an amount referenced to the amount of endogenous GARP2 present in these ROS membranes (see Experimental Procedures). For each experimental condition, catalytic activity was normalized to the PDE6 activity in the absence of exogenous GARP2 (defined as zero on the x-axis): 87 ± 19 (n = 9), 398 ± 105 (n = 5), and 4260 ± 680 (n = 4) cGMP hydrolyzed per PDE6 per sec for nonactivated PDE, transducin-activated PDE, and trypsinized PDE, respectively.

As seen in Fig. 3.5, addition of purified, native GARP2 to nonactivated PDE6 attached to ROS membranes (which contain endogenous GARP2) inhibited the basal rate of PDE6 activity by 80%. The suppression of PDE6 activity by GARP2 was maximal when an amount of purified GARP2 was added equal to its endogenous level in ROS (as determined by quantitative immunoblot analysis). A similar result was obtained with purified PDE6 that had been extracted from ROS membranes and chromatographically purified (data not shown).

In contrast, no significant effect of GARP2 on either transducin-activated PDE6 (attached to ROS membranes) or trypsin-activated PDE6 was seen (Fig.3.5). Even

following addition of a 10-fold excess of purified GARP2 relative to its endogenous concentration in ROS membranes, the PDE6 activity of both transducin- and trypsin-activated PDE6 remained within 20% of its activity in the absence of GARP2. While the results in Fig. 3.5 for trypsin-activated PDE6 are in accord with those of Korschen et al., we failed to observe the inhibitory effect of GARP2 on transducin-activated PDE6 that they reported (Korschen et al., 1999).

To more sensitively test whether GARP2 influences the ability of transducin to activate PDE6, we first supplemented light-exposed ROS membranes with either GARP2 or nothing, and then added increasing amounts of GTPγS to persistently activate transducin in a concentration-dependent manner.

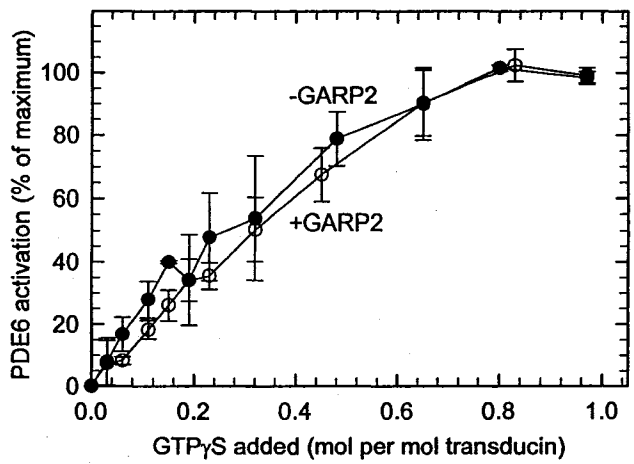

Figure 3.6. Inability of GARP2 to reduce activation of PDE6 by transducin. ROS homogenates (1.5 nM PDE6) were first incubated with either a 2-fold excess of GARP2 relative to its endogenous concentration (+GARP2) or buffer (-GARP2). The ROS membranes were exposed to light, and then the indicated amount of GTPγS was added to persistently activate transducin. The PDE6 activity is normalized to the extent of activation by saturating amounts of GTPγS, compared to the nonactivated PDE6 rate (150 or 110 cGMP per PDE6 per s in the absence or presence of GARP2, respectively). The data represent the mean ± S.E.M. (n = 3).

Fig. 3.6.(Rechsteiner and Rogers, 1996) shows that in the presence of GARP2 (at a concentration two-fold greater than required to maximally suppress PDE6 basal activity), the ability of transducin to activate PDE6 is only slightly impaired; the small decrease in activation at any given GTPγS concentration was not statistically significant. Once sufficient GTPγS was added to activate all of the transducin present (the transducin:PDE6 ratio in bovine ROS being 30:1), adding more GTPγS had no further effect on PDE6 activation in the absence or presence of GARP2.

This result shows that the activity-lowering effect of GARP2 on the nonactivated PDE6 holoenzyme is distinct from the molecular events by which the activated α-subunit of transducin binds to PDE6 and displaces its Pγ subunit, thereby causing light activation of PDE6.

Proposed physiological role for GARP2 regulation of PDE6 in rod photoreceptors—In this study, we have shown that GARP2 is a high-affinity PDE6 regulatory protein capable of suppressing the basal activity of nonactivated PDE6, but with negligible effects on transducin-activated PDE6. The exclusive localization of GARP2 to rod outer segments (Colville and Molday, 1996; Korschen et al., 1999) suggests that this protein may regulate rod PDE6 in a way that helps distinguish the rod and cone phototransduction pathways. One feature that differentiates rods from cones is the amplitude of fluctuations in the dark current ("dark noise") in the photoreceptor outer segment (Rieke and Baylor, 1996; Rieke and Baylor, 2000). The low dark noise of rods permits reliable signaling at the single photon level (Baylor et al., 1979), whereas cones require several photons to generate a detectible signal (Schnapf et al., 1990).

Because the rates of PDE6 activation/inactivation determine the characteristics of photoreceptor dark noise (Holcman and Korenbrot, 2005), GARP2 is an attractive candidate for regulating rod PDE6 to lower its spontaneous activation. This study suggests that binding of GARP2 to nonactivated rod PDE6 will lower its catalytic activity, most likely by enhancing the affinity of Pγ for the active site of the enzyme. The observed localization of GARP2 to the rim of the disk membrane in ROS (Colville and Molday, 1996; Korschen et al., 1999) may serve as a mechanism to reduce spontaneous PDE6 activation and thereby minimize fluctuations in cGMP concentrations in the vicinity of the cGMP-gated ion channel.

The lack of an effect of GARP2 on transducin-activated PDE6 is also consistent with the need for rod PDE6 to be rapidly and stoichiometrically activated upon binding of activated transducin. The single photon sensitivity of rod photoreceptors would likely be impaired if GARP2 were to reduce the efficiency of PDE6 activation by transducin. In summary, our results support a role for GARP2 in maintaining a very low spontaneous activation of PDE6 without interfering with the efficiency of the visual excitation pathway in rod photoreceptors in response to photic stimuli. Reports that GARP2 is associated with the disk rim protein, peripherin (Poetsch et al., 2001; Batra-Safferling et al., 2006), leave open the intriguing possibility that GARP2 might preferentially regulate PDE6 in the vicinity of the cGMP-gated channel where cGMP ,metabolic flux might be most stringently controlled.

Summary

This paper demonstrates that GARP2 is a novel PDE6 interacting protein that is capable of regulating the basal activity of the PDE6 holoenzyme in rod outer segments. The high affinity with which it binds nonactivated PDE6 suppresses catalytic activity without adversely affecting the ability of transducin to activate PDE6. This novel regulatory mechanism may be of fundamental importance in establishing the high signal-to-noise ratio needed for single photon detection in rod photoreceptors. Future studies will be directed toward determining the molecular mechanism of GARP2 interaction with the catalytic and/or inhibitory subunits of dark-adapted and light-activated PDE6.

CHAPTER 4

MULTIPLE SITES OF INTERACTION OF GARP2 WITH PDE6 HOLOENZYME AND MECHANISM OF REGULATION[6]

Abstract

Rod photoreceptors are unique in their ability to detect a single photon of light. However, at high light intensities, rod photoreceptor photoresponses are saturated and under these conditions electrical responses are generated only by cone photoreceptors. Some of the differences in the physiological responses of rods and cones can be attributed to proteins expressed exclusively in one type of photoreceptor cells. Previously, it has been shown that rods contain a class of proteins not found in cones, specifically the Glutamic Acid-Rich Proteins (GARPs). One of these proteins, GARP2, interacts with

[6] The abbreviations used are: PDE, phosphodiesterase (E.C. 3.1.4.35); PDE6, retinal photoreceptor PDE; PDE$\alpha\beta$, catalytic heterodimer of rod PDE6; Pγ, inhibitory 10 kDa subunit of rod PDE6; GARP, glutamic acid-rich protein; rGARP2, recombinant glutamic acid-rich protein 2; Tα, α-subunit of the rod photoreceptor G-protein, transducin; ROS, rod outer segment; SDS-PAGE, sodium dodecil sulfate polyacrylamide gel electrophoresis; nPDE, nonactivated PDE6; tPDE, trypsin-activated PDE6; taPDE, transducin-activated PDE6; GTPγS, guanosine 5'-O-[3-thiotriphosphate]; IPTG, isopropyl-beta-D-thiogalactopyranoside.

high affinity with PDE6, and lowers the basal rate, or "dark noise" of rod photoreceptors, contributing to the high light sensitivity of these cells. However, GARP2 does not have an effect on the ability of transducin to activate PDE6. Here we show that GARP2 interacts with PDE6 only at specific stages of the activation-inactivation cycle of PDE6. GARP2 is able to influence the PDE6 basal rate by increasing the inhibitory PDE-gamma subunit affinity for the PDE6 catalytic dimer. We propose that GARP2 interacts at several sites on the PDE6 holoenzyme to affect allosteric control of PDE6 activity in the dark-adapted, but not light-adapted state.

Introduction

The presence of the Glutamic Acid-Rich Proteins (GARPs) in rods and their absence in cones (Korschen et al., 1999; Colville and Molday, 1996) suggest an unique role for GARPs in the physiological response of rod photoreceptors (see Chapter 1) versus cones response. The GARP family of proteins consists of the β-subunit of the cGMP-gated channel, and two splice variants: GARP1 and GARP2. All three members of the GARP family are alternative transcripts the same gene, located on human chromosome 16 (Ardell et al., 1996). Recent studies show that GARP2 has a natively unfolded structure and exists in a monomer-multimer equilibrium (Batra-Safferling et al., 2006). These physical characteristics suggest that GARP2 might have a role in maintaining the structure of rod photoreceptor outer segment, serving as a linker between the plasma membrane and the disk rims (Batra-Safferling et al., 2006). GARP2 has four proline-rich regions (Colville and Molday, 1996; Korschen et al., 1995) that might be responsible for interacting with other proteins of the rod outer segment (Korschen et al., 1999). However, it appears that GARP2 makes high-affinity interactions only with PDE6 (Korschen et al., 1999; Pentia et al., 2006) and with peripherin-2 (Poetsch et al., 2001). Interaction with peripherin-2 might serve for anchoring the plasma membrane channel to the rod disks (Poetsch et al., 2001). The high content of glutamic acid, and the presence of the glutamic acid-rich region in GARP1 and the β subunit of the cGMP-gated channel confer to all GARPs their abnormal electrophoretic mobility (Sugimoto et al., 1991; Ardell et al., 1995; Colville and Molday, 1996).

We demonstrated in a previous study that GARP2 interacts with PDE6 with high affinity, and upon this interaction the basal activity, or the "dark noise" of PDE6 is reduced (Pentia et al., 2006). However, we could not detect any influence of GARP2 on activated PDE6. This finding might suggest that GARP2 exerts its effect by interacting with the inhibitory Pγ subunit of PDE6, since the activation of PDE6 requires the removal of the inhibition by Pγ. We hypothesize that GARP2 enhances the affinity of the inhibitory Pγ subunit to PDEαβ, lowering the PDE6 basal rate and conferring rod photoreceptors greater sensitivity for light stimulus.

Enzymatic catalysis of PDE6 is regulated by its inhibitory Pγ subunit. The interaction between the inhibitory subunit Pγ and PDEαβ is complex, with multiple sites of interaction (Artemyev and Hamm, 1992; Artemyev et al., 1996a; Granovsky et al., 1997; Granovsky et al., 1998; Natochin and Artemyev, 1996). The N-terminal half of Pγ contains a polycationic region (amino-acids 20-45) which makes a high-affinity interaction with the cGMP binding GAF domain of PDEαβ (Artemyev and Hamm, 1992; Lipkin et al., 1993; Natochin and Artemyev, 1996). This interaction is stronger when the GAF domains are occupied by cGMP, and weaker when they are empty (Mou and Cote, 2001). The C-terminus region of Pγ makes direct contact with the catalytic domain of PDEαβ imparting the inhibitory effect on catalysis (Skiba et al., 1995; Granovsky et al., 1997). There is no evidence that allosteric binding of cGMP to the regulatory GAF domains directly regulates the catalytic activity of PDE6. However, the binding affinity of Pγ to the PDEαβ is allosterically regulated by the occupancy of the GAF domains by cGMP (Cote et al., 1994; Yamazaki et al., 1982; Norton et al., 2000; Mou et al., 1999; Mou and Cote, 2001).

Membrane-associated PDE6 holoenzyme has two high-affinity cGMP binding sites (Gillespie and Beavo, 1989). The two sites however are not identical in their binding affinity for cGMP, one site becoming exchangeable and decreasing its binding affinity by more than 100 fold upon the removal of Pγ, while the second cGMP binding site has only a slightly lower binding affinity (Mou et al., 1999). It appears that Pγ binding to PDEαβ occurs as a two step process, one Pγ binds with high affinity ($K_D < 0.3$ pM), while the second Pγ affinity is much lower ($K_D = 7$ pM). It is hypothesized that the high affinity Pγ binding site is correlated with the high affinity cGMP binding site at the GAF domains (Mou and Cote, 2001). Pγ also makes interactions with Tα-GTP at the C-terminal region of Pγ (amino-acids 77-87) and at the poly-cationic region (Artemyev et al., 1992; Artemyev and Hamm, 1992; Slepak et al., 1995; Slep et al., 2001). The C-terminal region of Pγ has a much lower affinity for PDEαβ (Mou and Cote, 2001). The C-terminus of Pγ can interact with the GTPase activating protein RGS9 at the amino-acid Trp70 (Slep et al., 2001; Tsang et al., 1998; Slepak et al., 1995). Following this interaction, the GTPase activity of RGS9 is enhanced, Pγ serving as a GTPase activating protein (GAP; (Arshavsky et al., 1994; Angleson and Wensel, 1993).

In its non-activated state, PDEαβ catalytic sites are blocked by two Pγ inhibitory subunits. PDE6 is activated by the binding of activated transducin (Tα-GTP) to the C-terminus region of the inhibitory Pγ subunit (Yamazaki et al., 1983; Yamazaki et al., 1990; Wensel and Stryer, 1990; Wensel and Stryer, 1986; Gray-Keller et al., 1990), and subsequent exposure of the catalytic site, enabling the hydrolysis of cGMP. Experimental evidence suggests that Tα-GTP can only activate one catalytic site of the PDE6 heterodimer (Melia et al., 2000; Norton et al., 2000). Following activation, the cGMP

level concentration drops, increasing the likelihood that the cGMP bound to the GAF domains will dissociate (Mou et al., 1999). The relative binding affinity of Pγ-Tα-GTP complex for PDEαβ decreases upon cGMP release from the GAF domain, allowing the dissociation of Pγ-Tα-GTP complex (Cote et al., 1994). This dissociation may promote the GTPase activating protein (GAP) activity of Pγ (Arshavsky and Bownds, 1992). Upon Tα deactivation, Pγ is released and is able to re-inhibit PDEαβ and revert to the non-activated state. The cycle of PDE6 activation and inactivation is summarized in Fig. 4.1.

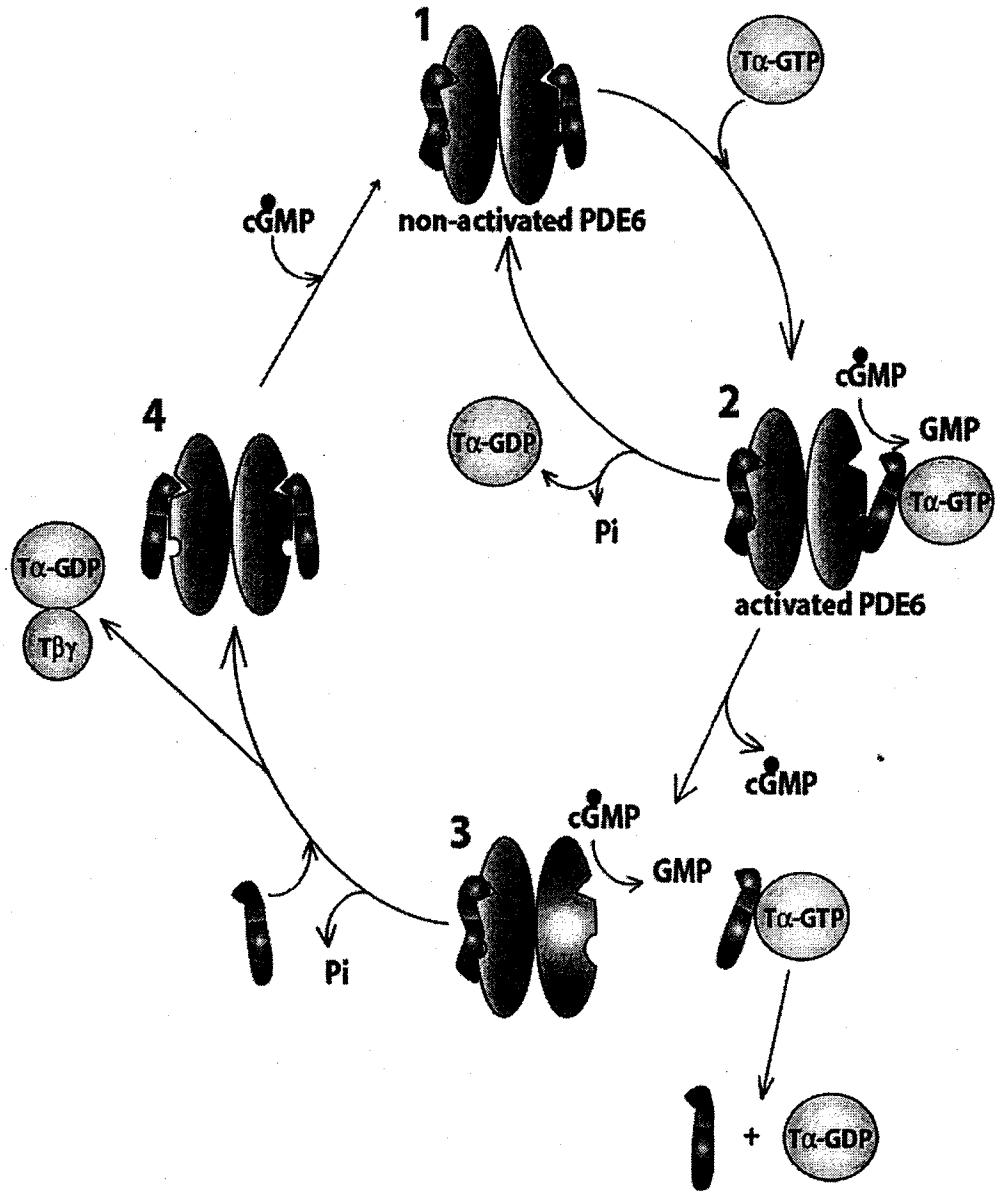

Figure 4.1. PDE6 activation-deactivation cycle.

State 1. Dark-adapted state: PDE6 has two Pγ subunits bound, and the GAF domains are occupied by cGMP.

State 2. Initial transducin-activated state: Tα-GTP interacts with Pγ and releases inhibition at one catalytic site. cGMP still occupies the GAF domains.

State 3. Persistent transducin-activated state: as cGMP levels remain low, one GAF domain rapidly releases cGMP (the second GAF domain releasing bound cGMP more slowly). This enhances release of Tα-GTP-Pγ from PDE6. Subsequently, GTP is hydrolyzed to GDP and Pγ dissociates from Tα-GDP.

State 4. Pγ re-associates with PDEαβ, but cGMP levels are still low and GAF domains remain empty until cGMP levels are restored to dark levels.

The goal of this study is to identify the steps of the PDE6 activation-deactivation cycle that are able to bind GARP2 and the sites of interaction of GARP2 with PDE6 holoenzyme. We hypothesize that GARP2 interaction takes place primarily with Pγ, enhancing its affinity to bind to PDEαβ and thereby reducing the probability of spontaneous activation of non-activated PDE6 and lowering the basal rate of PDE6 activity (Pentia et al., 2006). We further speculate that efficient activation of PDE6 by transducin requires dissociation of GARP2 from PDE6 upon light activation. Therefore we analyze the interaction sites of GARP2 with PDE6 holoenzyme during the different activation conditions.

Materials and Methods

1. Materials:

Bovine retinas were purchased from W. L. Lawson Co, Lincoln, NE. [^{3}H]cGMP was purchased from PerkinElmer Life Sciences. Ultima-Gold scintillation fluid was obtained from Perkin Elmer. Membranes for filter binding assays were purchased from Millipore. Reagents and substrates for immunoblots were from Pierce and Bio-Rad. 6-His-tagged recombinant GARP2 plasmid was a kind gift from Dr. Benjamin Kaupp (Julich, Germany). All other chemicals were obtained from Sigma. Antibodies used were: anti-NC, N-terminal half of PDE6 catalytic subunit; anti-CT, C-terminal Pγ; anti Tα, from Affinity Bioreagents; and anti-GARP2, a kind gift of Dr. Benjamin Kaupp.

2. Expression and purification of recombinant GARP2.

The GARP2 protein used in these studies was expressed as a 6His-flagged recombinant protein in *E. coli* cells. The rGARP2 plasmid, a kind gift for Dr. Benjamin Kaupp (Julich, Germany), was cloned between restriction sites EcoRI and HindIII of the pET30a vector. The vector was transformed in *E.coli* BL21 competent cells following standard procedures. Glycerol stocks were generated from the transformed cells. Initial colonies of plasmid containing *E.coli* cells were grown from the glycerol stock on a kanamycin plate. A single colony from the plate was inoculated in a LB medium containing 50 μg/ml kanamycin, and incubated with shaking overnight at 37°C. The following morning, 10 ml from the overnight culture were inoculated into two 500 ml 2xTY media also supplemented with 50 μg/ml kanamycin. The cells were incubated with shaking at 20°C for 4 hours, until the OD_{600} reached 0.6-0.8. Protein expression was then

induced by addition of 1 mM IPTG. Additional 3 hours incubation at 20°C allowed the expression of full-length GARP2 protein. These expression conditions were found to be optimal for obtaining full-length rGARP2 that runs on SDS-PAGE at an apparent MW of 60kDa. A higher incubation temperature produces truncated proteins (see Fig. 4.2.).

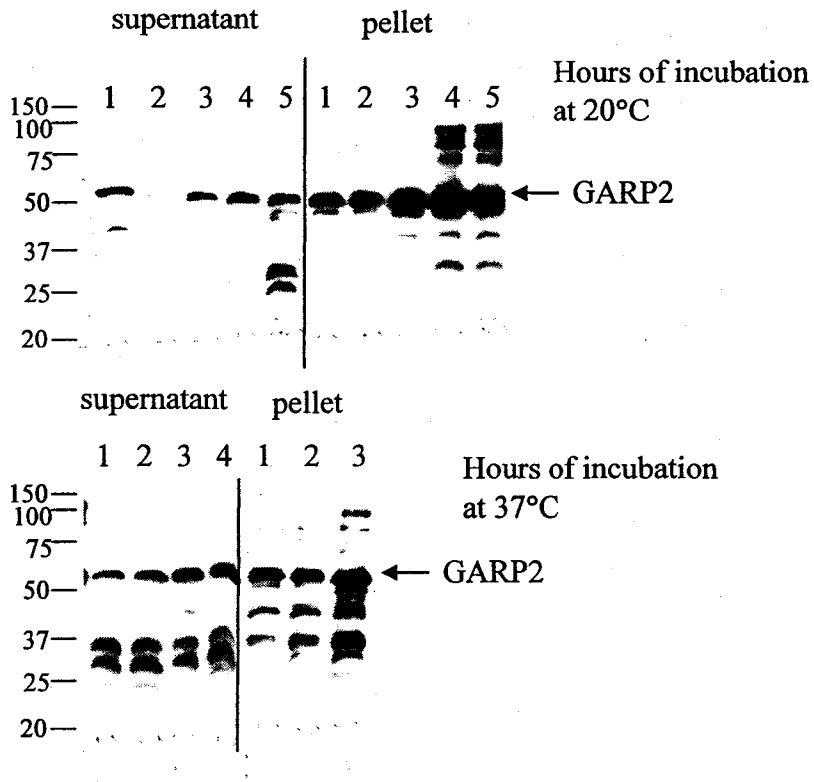

Figure 4.2. Conditions for optimal expression of rGARP2.
E. coli cells harboring the rGARP2 plasmid were tested for rGARP2 expression by incubating at 20°C and 37°C for various times. The cells were then lysed and the supernatants and pellets were analyzed by western blot using full-length anti-GARP2 antibody.

Cells were pelleted by centrifugation and resuspended in His-Bind binding buffer supplemented with EDTA-free protease inhibitors. To disrupt the cells, two freeze- thaw cycles followed by two sonication bursts were used. The lysate was centrifuged for 30 min at 100,000 x g, and the supernatant was recovered.

Recombinant GARP2 was purified from the cells lysate by Ni-chelated affinity method. The His-Bind resin (Novagen) was charged with a solution of 50 mM $NiSO_4$. The column was equilibrated with 3 column volumes of binding buffer (0.5 M NaCl, 20mM Tris-HCl, pH 7.9). The bacterial extract was applied to the column at a flow rate of 0.5 ml/min. The unbound material was washed with 10 column volumes of binding buffer at a flow rate of 1 ml/min. Bound proteins were eluted with 2 column volumes of elution buffer (0.5 mM NaCl, 20 mM Tris-HCl, pH 7.9, 150 mM imidazole). Further purification of recombinant GARP2 was required. The protein eluted from the His-Bind column was dialyzed overnight in a low salt buffer (5 mM Tris, pH 7.5, 100 mM NaCl, 1 mM DTT). The rGARP2 solution was applied to the MonoQ column, the unbound proteins were washed with 5 column volumes of low salt buffer, and the bound proteins were eluted with a linear gradient from 100-1000 mM NaCl in 5 mM Tris, pH 7.5. 1 ml fractions were collected. The rGARP2 peak was detected by dot-blotting the fractions on nitrocellulose membrane, and probing the membrane with anti-GARP2 C-terminus antibody (Affinity Bioreagents).

2. PDE6 activity assays.

2.1 Colorimetric phosphate release assay.

The rates of cGMP hydrolysis catalyzed by activated and non-activated PDE6 were measured by a coupled-enzyme phosphate release assay (Cote, 2000).

2.2 Radiotracer assay.

The assay is a coupled enzyme assay involving determination of the radioactive [^{3}H]guanosine that is generated by hydrolysis of [^{3}H]cGMP by PDE6 which yields 5'-

[^{3}H]GMP. Addition of snake venom to the assay solution causes the 5'-[^{3}H]GMP to be degraded to [^{3}H]guanosine plus inorganic phosphate. Separation of [^{3}H]guanosine from un-reacted [^{3}H]cGMP occurs by passage of the sample through an anion-exchange column (DEAE-Sephadex) equilibrated with a low salt solution (20 mM Tris-HCl, pH 6.8). This assay is much more sensitive than the colorimetric phosphate release assay, and it gives good estimates for non-activated PDE activity. It is also suitable for very low (pM) concentrations of activated PDE6.

3. Preparing GARP2-free ROS membranes.

Sucrose purified bovine ROS membranes were resuspended in the dark in isotonic buffer (10 mM Tris, pH 7.5, 100 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT), and centrifuged for 5 min at 130,000 x g to remove the soluble proteins. At this point, the ROS membranes were exposed to light to ensure the tight binding of Tα to the membranes. The membranes were resuspended in the same isotonic buffer as above, supplemented with 0.1% v/v Triton X-100. The solution was centrifuged for 5 min in the Airfuge at 130,000 x g. The supernatant contained all the GARP2, while the pellet contained PDE6 and Tα (Fig. 4.3.). The pellet was washed two times with isotonic buffer and centrifuged as above in the Airfuge to remove any trace of detergent.

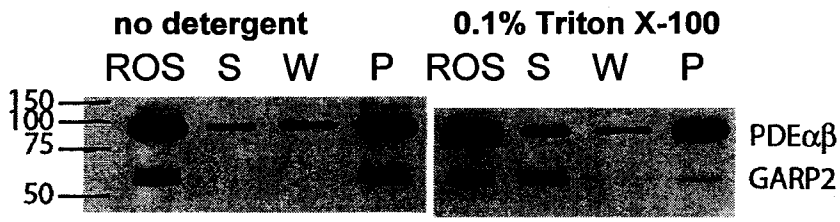

Figure 4.3. Preparation of GARP2-free ROS membranes.
ROS membranes were treated with 0.1% Triton X-100 and the soluble portion (S) was separated from membranes (P) by centrifugation for 5 min at 130,000 x g. The control sample was treated with isotonic buffer. Following detergent treatment, the membranes were washed of residual detergent with isotonic buffer (W). Resulting samples were analyzed by western blot using anti- full-length GARP2 antibody and anti- catalytic subunit PDE6 antibody.

4. Preparation of recombinant PγFL, Pγ1-45, and Pγ63-87.

Full-length bovine rod Pγ and the N-terminal fragment consisting of the first 45 amino-acids (Pγ1-45) were expressed in *E. coli* cells using a pET11a expression vector (Slepak et al. 1995) and purified as described elsewhere (Artemyev et al., 1998). Briefly, the bacterial extract was purified on a cation exchange column (SP-Sepharose, GE Healthcare), and the protein of interest (Pγ or Pγ1-45) was chromatographed on a semi-preparative C4 reverse-phase column (300 Å, 10 x 250 mm) using a linear gradient of 30-80% acetonitrile in 0.1% trifluroacetic acid. The concentration of full-length Pγ was routinely measured spectrophotometrically ($\varepsilon_{277} = 7550\ cm^{-1}\ M^{-1}$; (Cote, 2000). The concentration of purified Pγ1-45 was measured using the bicinchoninic acid protein assay (Smith et al.,1985). Pγ63-87 was purchased commercially, and purified by reverse-phase high pressure liquid chromatography on a preparative C4 column (Vydac), using a linear gradient of 30-80% acetonitrile. After lyophilization, the peptide was resuspended in 10 mM Tris, pH 7.5, and its concentration determined by the bicinchoninic acid protein

assay (Smith et al., 1985) using bovine serum albumin as the standard. The inhibitory capacity of purified Pγ was verified by PDE6 activity assays.

5. Pull-down assay of Pγ–interacting proteins with Pγ beads.

Pγ peptides and full length Pγ were prepared as described above. The proteins were coupled to CNBr-activated Sepharose according to the manufacturer's protocol (GE-Healthcare). rGARP2 (10 μL of 100 μM) was incubated with 10 μL of settled beads in a final volume of 200 μL of TMN buffer (50 mM Tris, pH 7.5, 140 mM NaCl, 0.5 mM $MgCl_2$, 0.2 mM Pefabloc) for 4 hours at 4°C with rocking. After the incubation, the supernatant was spun and removed, and the beads were washed two more times with 1 mL of ice-cold TMN buffer. After the washes, the beads were treated with 20 μL of gel sample buffer, heated for 5 min at 85°C, and the extract removed by centrifugation. The collected samples were run on a 12% acrylamide SDS-PAGE, transferred to nitrocellulose membrane, and probed with the relevant antibodies.

6. Measurements of cGMP binding to the non-catalytic GAF domains of PDE6.

A membrane filtration assay was used to determine the amount of radiolabeled cGMP bound to the non-catalytic sites of PDE6 (Cote, 2005). Given the high affinity nature of cGMP binding to PDE6 (K_D<μM) (Cote et al., 1994; Cote and Brunnock, 1993) this method offers high sensitivity, good partitioning of free from bound ligand, and low non-specific binding. Some precautions needed to be taken. First, all the endogenous cGMP that was bound to the GAF domains was destroyed by incubating PDEαβ at 30°C for 4 hours. Because cGMP is both a ligand for the non-catalytic GAF domains and

substrate for the catalytic domain, a second precaution was to block the catalytic sites by using a selective inhibitor (zaprinast) at 1 mM. The binding reaction was initiated by addition of [^{3}H]cGMP solution to the depleted PDE$\alpha\beta$. Immediately following cGMP addition, Pγ was added to some samples stabilize the low affinity cGMP binding site. Samples were applied to pre-wet nitrocellulose membranes over a vacuum filtration device, and unbound ligand was rinsed with 3 x 1 ml washes of isotonic buffer (100 mM Tris, pH 7.5, 1 mM EDTA). The membranes were placed in scintillation vials, mixed with scintillation fluid and the total counts were measured in the scintillation counter.

7. Preparation of lipid rafts from bovine ROS membranes.

Purified ROS membranes were resuspended in an isotonic buffer (10 mM MOPS, pH 7.2, 60 mM KCl, 30 mM NaCl, 5 mM $MgCl_2$, 1mM DTT). The samples used for analyzing lipid domains in the non-activated state were kept in the dark for the entire duration of the experiment; otherwise, the ROS membranes were exposed to light, and treated with the required nucleotides. The resuspended ROS samples were treated with Triton X-100, (final concentration of 1% v/v). The ROS membranes were then homogenized with 10 passes of a glass homogenizer. The homogenized ROS membranes were incubated on ice for 10 minutes, and adjusted to 0.9 M sucrose using a 2.4 M sucrose solution. The sucrose ROS solution was placed on the bottom of a 17 ml ultra-centrifuge tube, and was overlaid with sucrose solutions of the following concentrations: 0. 8 M, 0.7 M, 0.6 M and 0.5 M. The tubes were placed centrifuged for 20 hours at 100,000 x g. At the end of the spin, the Triton X-100 insoluble lipid domains separated at the interface of the 0.5 M and 0.6 M sucrose layers. Fractions of 500 µL were collected

from the top of the tube. Fractions were analyzed for PDE6 activity using a colorimetric assay and rhodopsin concentration was measured by monitoring light absorbance of each fraction at 500 nm (Bownds et al., 1971). Detergent-insoluble lipid domains were also detected from the turbidity of the samples by monitoring light absorbance at 600 nm. Proteins of interest (GARP2, PDE6, Tα) were detected by western blot analysis.

Results and Discussion

1. GARP2 enhances Pγ affinity for PDEαβ

Our previous study showed that GARP2 is able to reduce the spontaneous activation of PDE6, thereby reducing the basal rate of PDE6 activity (Pentia et al., 2006). One possible mechanism by which GARP2 has this effect is by enhancing the binding affinity of the inhibitory Pγ subunit for PDE6. It is more likely that GARP2 inhibitory effect takes place through Pγ than by direct inhibition of GARP2 on PDE6 catalysis, because GARP2 is not able to inhibit PDEαβ catalytic dimer by itself. There is no observed inhibition on either trypsinized PDE6 or transducin-activated PDE6 (Pentia et al., 2006). In order to determine whether GARP2 effect on catalysis is a result of the altered affinity of Pγ to PDEαβ, Pγ affinity was measured in the presence and absence of GARP2.

1.1. Preparation of GARP2-free ROS membranes.

To more closely mimic the physiological situation in which PDE6 is membrane associated, these experiments were carried out using purified bovine ROS membranes diluted in an isotonic buffer (see Materials and Methods). To determine the effect of GARP2 on Pγ affinity for PDEαβ, ROS membranes had to be stripped of their endogenous GARP2. We developed a protocol that selectively removed GARP2 from ROS membranes, using low concentrations of the detergent Triton X-100. Several concentrations of detergent were initially tested (see Fig. 4.4.), ranging from 0.1% to 1% v/v Triton X-100. GARP2 was released almost completely even at 0.1% Triton X-100.

As the concentration of detergent increased, PDE6 started to be released also. The concentration of 0.1% Triton X-100 was chosen to elute GARP2 from ROS membranes without elution of significant amounts of PDE6. After the removal of soluble GARP2 in detergent, the ROS membranes were washed two times in isotonic buffer to remove any trace of detergent and contaminating GARP2.

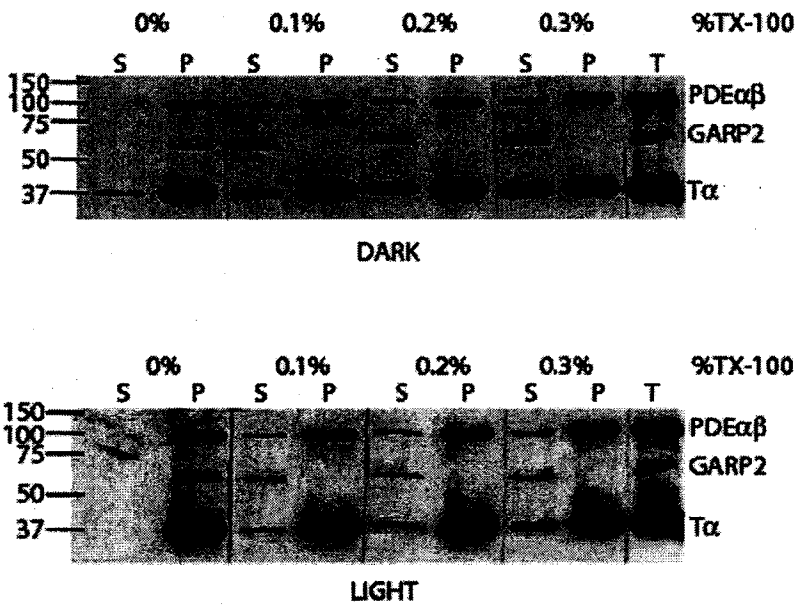

Figure 4.4. Determining conditions that release endogenous GARP2 from ROS membranes with minimum loss of PDE6 or Tα. Light-exposed ROS membranes were washed first with isotonic buffer and centrifuged for 5 min at 130,000 x g to remove soluble proteins. The resulting pellets were incubated with the indicated concentrations of the detergent Triton X-100 for 5 min at room temperature, and centrifuged again to separate the detergent-soluble fraction (S) from the pellet (P). The resulting samples were resuspended in gel sample buffer, and equivalent volume samples were subjected to SDS-PAGE. The separated proteins from the gel were transferred to nitrocellulose membrane, and separately identified with specific antibodies: anti-PDE_{NC}, anti-FL-GARP2 and anti-Tα.

1.2. Measuring the binding affinity of Pγ to PDEαβ.

Previous work showed that dilution of PDE6-containing frog ROS membranes could be used as a way to estimate the K_D of Pγ binding to PDEαβ (D'Amours & Cote 1999). To measure the Pγ affinity for bovine PDEαβ and the effect of GARP2, Pγ dissociation was induced by diluting the bovine PDE6 holoenzyme, thereby shifting the binding equilibrium towards dissociation:

$$PDE\alpha\beta + 2P\gamma \leftrightarrow PDE\alpha\beta\gamma\gamma$$

The apparent affinity of bovine Pγ to PDEαβ is 10 pM (Wensel and Stryer, 1986) while the K_D for frog Pγ is 28 pM (D'Amours and Cote, 1999). The PDE6 concentration chosen for these experiments was in the range of 1 nM-10 pM. This range of concentrations allows Pγ to dissociate, and the catalytic site to be exposed. The degree of dissociation can be monitored by the increase in PDE6 catalytic activity, the greater the activity, the more Pγ dissociates from PDEαβ. However, at concentrations lower than ~5 pM, PDE6 activity can not be measured reliably, probably because of the enzyme degradation during long incubation times that are required for such low concentrations. The high binding affinity of Pγ for PDEαβ of bovine PDE6, and the instability of the PDE6 at low concentrations made it difficult to observe the desired dilution effect. When frog ROS was used, the activation of PDE6 was far greater than when bovine ROS was used (see Fig. 4.5.). This comparison is direct experimental evidence that the affinity of Pγ for PDEαβ is greater for bovine ROS than it is for frog ROS. The activation of PDE6 for frog ROS was ~50% of V_{max} at the lowest concentration tested (5 pM) while at the same

concentration bovine ROS could only be activated up to 2%. Even so, a modest increase in activity is observed in the ROS samples that lack endogenous GARP2 compared to those that have not been depleted of GARP2.

To definitively determine that GARP2 indeed enhances the binding affinity of Pγ to PDEαβ, a greater activity is needed to be observed for the dilution of ROS membranes.

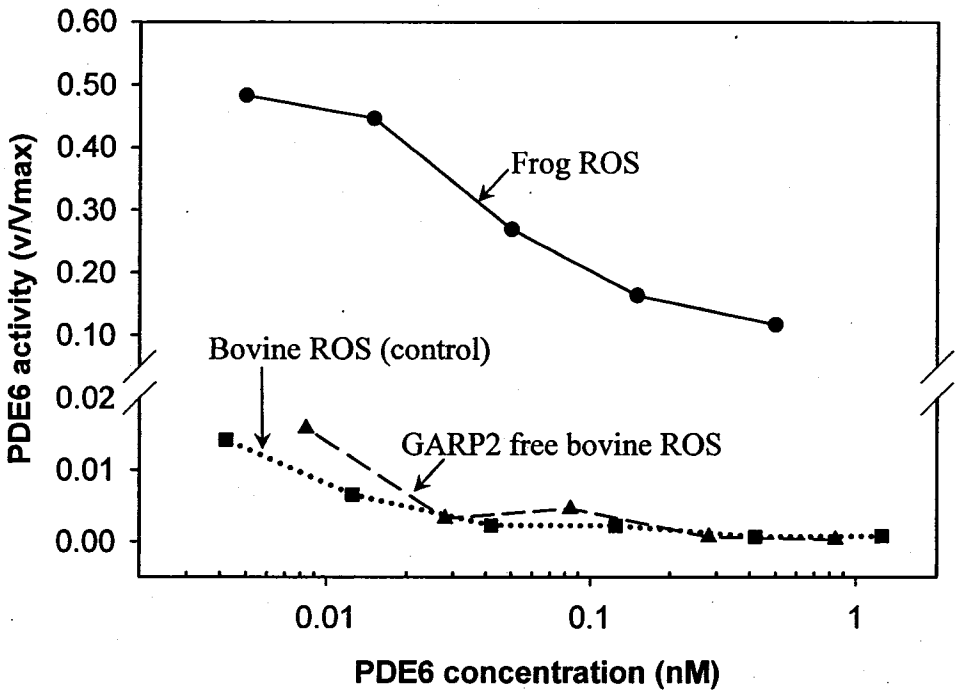

Figure 4.5. Comparison of frog and bovine ROS Pγ affinity for PDEαβ. Purified ROS were first washed with an isotonic buffer to remove the soluble proteins. For bovine ROS, one sample was treated with 0.1% Triton X-100 to remove endogenous GARP2. That ROS sample was washed two more times with isotonic buffer to remove traces of detergent. PDE6 concentration was determined for each ROS sample using an enzymatic assay. Samples were diluted to the indicated concentrations in isotonic buffer, and were incubated with 1 mM radiolabeled cGMP to analyze PDE6 activity with the readiotracer assay. The rate was normalized to the value expected for fully activated PDE6 (v/Vmax).

1.3. Using histones to destabilize Pγ binding to PDEαβ.

Other research groups have reported the activation of PDE6 using histones (Hurwitz and Beavo, 1984; Miki et al., 1975). Histones are also used for enhancing cGMP binding to the GAF domains of PDE5 (Corbin et al., 2003; Gopal et al., 2001; Weeks et al., 2005). The mechanism by which histones are able to bind to and regulate PDE is not well understood. It is hypothesized that for PDE6, negatively charged histones interact with the poly-cationic region of Pγ and displace Pγ from PDEαβ.

Upon addition of histone type II-AS the maximal dilution-induced activation of PDE6 increased by approximately 10-fold (see Fig. 4.6.).

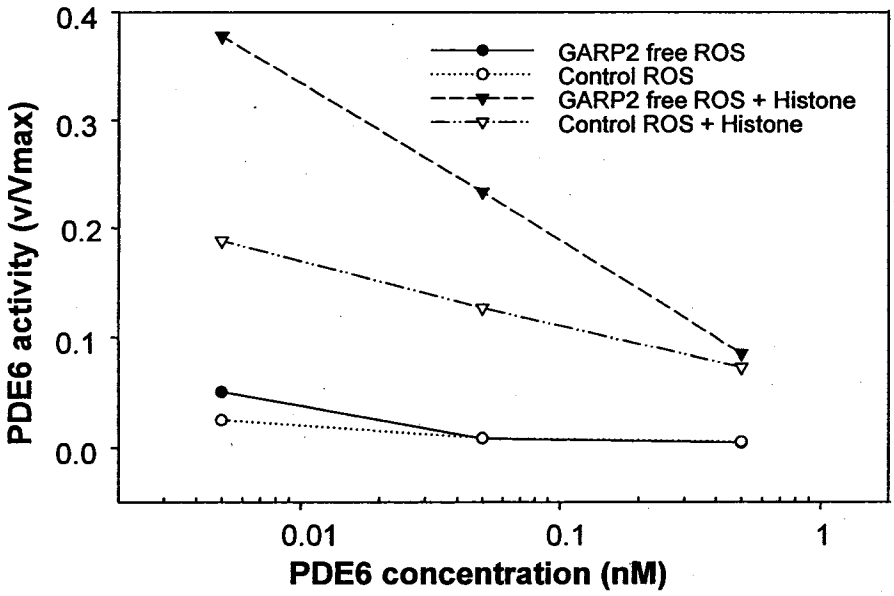

Figure 4.6. Improving conditions for Pγ dissociation from PDEαβ upon dilution. Bovine ROS membranes were first washed with isotonic buffer to release soluble proteins. Half of the samples were treated with 0.1% Triton X-100 to release endogenous GARP2, the other half being re-suspended in isotonic buffer. Samples were washed two more times in isotonic buffer. PDE6 concentration was determined using an enzymatic assay. Portions of each ROS sample were supplemented or not with 2.5 mg/ml histone type II-AS. Samples were incubated with 1 mM radiolabeled cGMP to determine PDE6 activity. The experiment is representative of four experiments done under similar conditions.

A concentration of 15 pM was chosen for determining the effect of GARP2 on the Pγ dissociation from PDEαβ. Adding recombinant GARP2 to ROS membranes depleted of their endogenous GARP2 caused a decrease in catalytic activity (see Fig. 4.7.). The activity of the GARP2-depleted ROS membranes at 15 pM PDE6 concentration is ~70% of that of ROS membranes fully activated by trypsinization, and ~60% for the membranes that still have the entire pool of endogenous GARP2 (see Fig. 4.7.). As the recombinant GARP2 concentration is increased, the PDE6 activity decreases, with a more pronounced effect in the case of the ROS membranes that have been depleted of the endogenous GARP2, and less for those that contain the endogenous GARP2.

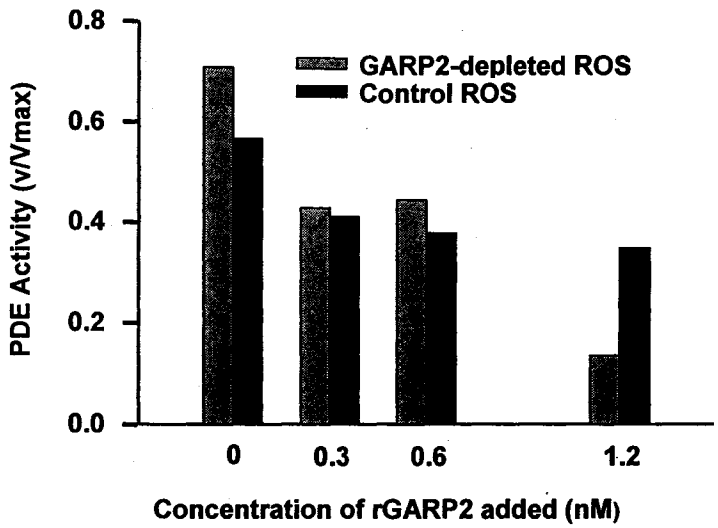

Figure 4.7. Addition of exogenous GARP2 enhances Pγ affinity.
The activity of 15 pM PDE6 holoenzyme was determined for control (containing endogenous GARP2) or GARP2-depleted ROS membranes to which the indicated amount of recombinant, purified GARP2 was added. PDE6 activity was measured using a radiotracer assay, and was expressed as percentage of the maximum rate for trypsin-activated PDE6 (Vmax = 5600 cGMP/s per PDE6). The experiment was performed once.

2. GARP2 regulates cGMP binding to the GAF domains of PDE6.

The effect of GARP2 on the catalytic activity of PDE6 holoenzyme suggested that GARP2 might also affect the cGMP binding to the regulatory GAF domains. As mentioned earlier, the regulatory GAF domains bind cGMP with different affinities: one site is a high affinity site binding cGMP with a K_D of ~ 60 nM, while the second cGMP binding site has a lower affinity (Mou et al., 1999). Upon Pγ interaction with PDEαβ, this low affinity cGMP binding site is able to convert to a high affinity binding site. Moreover, the binding of cGMP to the GAF domains also reciprocally regulates the affinity of Pγ binding: when the GAF domains are occupied by cGMP, Pγ has a higher binding affinity for PDEαβ (Mou & Cote 2001). Therefore, we hypothesized that GARP2 might not only modulate the affinity of the interaction between Pγ and the PDEαβ active site, but also affect the cGMP binding to the GAF domains.

To determine whether GARP2 influences the binding affinity of cGMP to the non-catalytic GAF domains of PDEαβ dimer, a filter binding assay was used. Briefly, PDE6 holoenzyme was mildly trypsinized to remove the inhibitory Pγ subunits. The resulting PDEαβ was incubated for 4 hours at 30°C to permit the endogenous cGMP that was bound to the GAF domains to dissociate and be hydrolyzed. The catalytic sites were then blocked by using a specific inhibitor (zaprinast 100 μM). A solution of 1 μM radiolabeled cGMP was added to 5 nM PDEαβ. The influence on the low affinity binding site was determined by adding recombinant Pγ in the absence or presence of recombinant GARP2. In a different set of experiments, the influence of GARP2 on the cGMP binding to the GAF domains was measured in the presence of recombinant Pγ1-45 peptide (the N-terminal half of Pγ that induces high-affinity interactions with the GAF domains).

In the absence of Pγ, cGMP binds to PDEαβ in a molar ratio of 1:1, as seen previously (Mou et al., 1999). The addition of GARP2 appears to lower somewhat the affinity of cGMP for the PDEαβ heterodimer (Fig. 4.8.). This suggests that GARP2 might modulate cGMP binding to the GAF domains by direct interactions with the PDEαβ dimer.

In the presence of Pγ, addition of rGARP2 reverses the increase in affinity for cGMP to the Pγ-dependent cGMP binding site. The same effect is observed if the N-terminus Pγ peptide is used (Pγ1-45) (data not shown). This finding suggests that GARP2 has a destabilizing effect on Pγ affinity for the sites on PDEαβ that are responsible for cGMP binding to the GAF domains. However, this is an indirect assessment of the Pγ affinity, based on the regulatory effect that Pγ has on cGMP binding to the GAF domains. In summary, these results would imply that GARP2 is able to interact with PDE6 holoenzyme at multiple sites of interaction. Some GARP2 interactions likely stabilize Pγ interaction with the active site, while other sites of interaction of GARP2 may destabilize cGMP binding to the GAF domains.

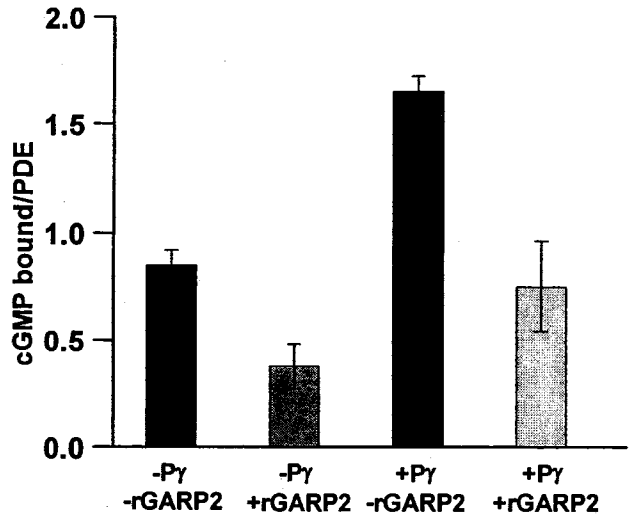

Figure 4.8. GARP2 reduces cGMP binding to a high-affinity site on Pαβ, and reverses the Pγ-dependent binding of cGMP to a second, lower affinity site. cGMP binding to the GAF domains of PDE6 catalytic dimer (5 nM) in the presence or absence of 70 nM Pγ and/or 5 nM recombinant GARP2 was measured with a membrane filtration assay. This result is representative of 4 different experiments.

3. Defining the sites of interaction of GARP2 with Pγ.

Previous experiments have determined that GARP2 influences PDE6 properties primarily by interacting with the inhibitory Pγ subunit. The reduction of PDE6 activity following the shifting in Pγ binding equilibrium to PDE$\alpha\beta\gamma_2$ could suggest that GARP2 interacts with the C-terminus part of Pγ, the part that interacts with the catalytic site on the catalytic subunit. Also the change in cGMP binding affinity to the GAF domains might suggest that GARP2 also interacts with the N-terminus part of Pγ, the part that makes direct contact with the GAF domains. Direct evidence of GARP2 interacting with Pγ is needed to determine that the interaction is indeed taking place.

A pull-down experiment was devised to directly determine if GARP2 is able to interact with full-length Pγ and Pγ fragments. The N-terminal Pγ peptide Pγ1-45, the C-

terminal peptide Pγ63-87 and full-length Pγ were purified as described in Methods. The peptides and protein were coupled to Sepharose beads and incubated with recombinant GARP2 for 1 hour. The beads containing Pγ binding proteins were washed with an isotonic buffer, and the protein complex was eluted with SDS-PAGE sample buffer. The proteins were analyzed on western blots probed with anti-GARP antibody.

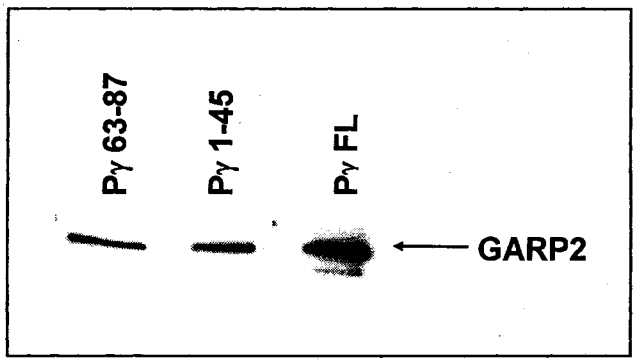

Figure 4.9. GARP2 is pulled down by Pγ and its N-terminal and C-terminal domains.
Purified GARP2 was incubated with full-length Pγ and the indicated Pγ peptides immobilized to CNBr-activated Sepharose beads. After centrifugation, the pellet was solubilized, loaded on SDS-PAGE, and an immunoblot performed using GARP2 antibody which recognizes the full-length protein. Control beads failed to pull down GARP2.

GARP2 was found to bind to both Pγ peptides, as well as to the full-length Pγ (Fig.4.9.). Previous experiments determined indirectly that GARP2 is able interact at multiple sites with the Pγ subunit of PDE6. The pull-down experiment is direct evidence that these interactions take place, and that GARP2 and Pγ interaction is a multiple region interaction. What is missing are controls showing no non-specific binding of GARP2 to

the beads in the absence of Pγ or fragments. The best control would be scrambled Pγ peptides to show specificity.

4. Separation of PDE6 and GARP2 during the light activation cycle.

The above findings suggest that GARP2 has an inhibitory effect on PDE6 by enhancing the Pγ affinity for PDEαβ. There is also the possibility that GARP2 interacts directly with the PDEαβ dimer. During light activation, however, activated transducin (Tα-GTP) interacts with the C-terminus of Pγ and removes the inhibitory constraint on the catalytic site. From electrophysiological and biochemical measurements (Pugh, Jr. and Lamb, 1993; Lamb and Pugh, Jr., 1992) it has been established that transducin activation of PDE6 is very efficient. In order to have a high efficiency of transducin activation, any GARP2 regulatory effect may need to be absent. Possible mechanisms that will contribute to reducing the GARP2 effect on PDE6 include: a competition between Tα-GTP and GARP2 for binding to the C-terminus of Pγ, or a separation of GARP2 from PDE6 and Tα-GTP upon activation. To test if GARP2 and PDE6-Pγ-Tα-GTP separate on the disk membrane, ROS membranes were separated using detergent conditions that generate lipid "rafts", and then the protein composition of PDE6-containing fractions was analyzed.

Lipid membranes are a fluidic mosaic of phospholipids, sphingolipids, cholesterol and proteins. Some portions of the membrane can assemble in to more structured domains, or lipid "rafts", which are structured domains enriched in cholesterol, sphingolipids, and are abundant in the protein caveolin, a marker for these lipid "raft" domains (Brown and London, 1998; Kurzchalia and Parton, 1999). Experimentally it has

been noticed that lipid "rafts" are insoluble in 1% Triton X-100 detergent, this constituting the method used for their separation.

In order to separate lipid "rafts" from ROS membranes, purified rod outer segments were treated with 1% Triton X-100, and then separated by a sucrose gradient centrifugation. Lipid "rafts", being more buoyant, were collected from a less dense sucrose layer (see Methods), while the detergent-soluble portions of the membrane were left in the most dense sucrose layer. Using this tool, lipid "rafts" separated from ROS membranes were analyzed, and the proteins of interest detected. The light exposure conditions for ROS membranes were controlled, so that one sample was kept in the dark for the duration of the "rafts" separation, while another sample was exposed to light and treated with the non-hydrolizable GTP analog, GTPγS, to induce a state of permanent activation. Upon separation of the membrane fractions, GARP2 was found only in the soluble parts of the ROS membranes, regardless of the state of activation of the membranes. PDE6 however showed a movement towards the detergent-insoluble membrane domains (or lipid "rafts") upon activation (Fig. 4.10.).

This result provides the first evidence that GARP2 and activated PDE6 may not be in close proximity to each other, preventing a potential "short-cut" of the activation pathway by GARP2's inhibitory effect on PDE6.

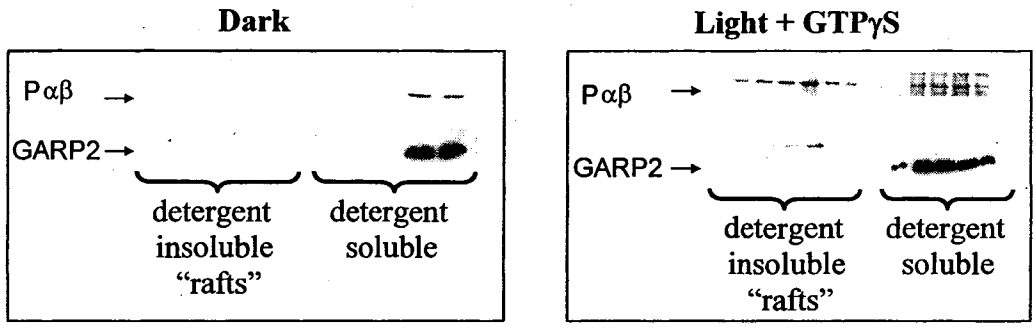

Figure 4.10. Lipid "raft" separation of ROS membranes. ROS membranes were homogenized in 1% Triton X-100 + 0.9 M sucrose and placed on the bottom of a sucrose step gradient: 0.9 M, 0.8 M, 0.7 M, 0.6 M and 0.5 M sucrose. After a 20 h centrifugation at 100,000 x g, fractions were collected from the top of the gradient. The lipid "rafts" are found at the interface of 0.5 M and 0.6 M sucrose. The bottom of the tube contains the detergent soluble, non-"raft" fraction of the membranes. For the dark condition, the experiment was conducted under infrared illumination. The light-activated ROS membranes were exposed to light and 1 mM GTPγS for transducin activation. Western blots detected the presence of PDE6 and GARP2 using the anti-catalytic subunit PDE6 antibody and the anti-GARP2 antibody. This is representative of 5 other experiments.

5. Regulation of GARP2 effect on PDE6 by dissociation of these binding partners upon light activation of PDE6.

Previously we showed that GARP2 can reduce the spontaneous activation of PDE6 in the dark, contributing to the reduced dark "noise" of rod photoreceptors and their enhanced light sensitivity. If GARP2 and PDE6 separate following transducin activation, there must be a later step of the activation-inactivation cycle of PDE6 that restores GARP2-PDE6 interaction in order for GARP2 to re-exert its inhibitory effect in the dark state. To study the association –dissociation states of GARP2 and PDE6 during the activation cycle, the different steps of the cycle had to be isolated (see Fig. 4.11.).

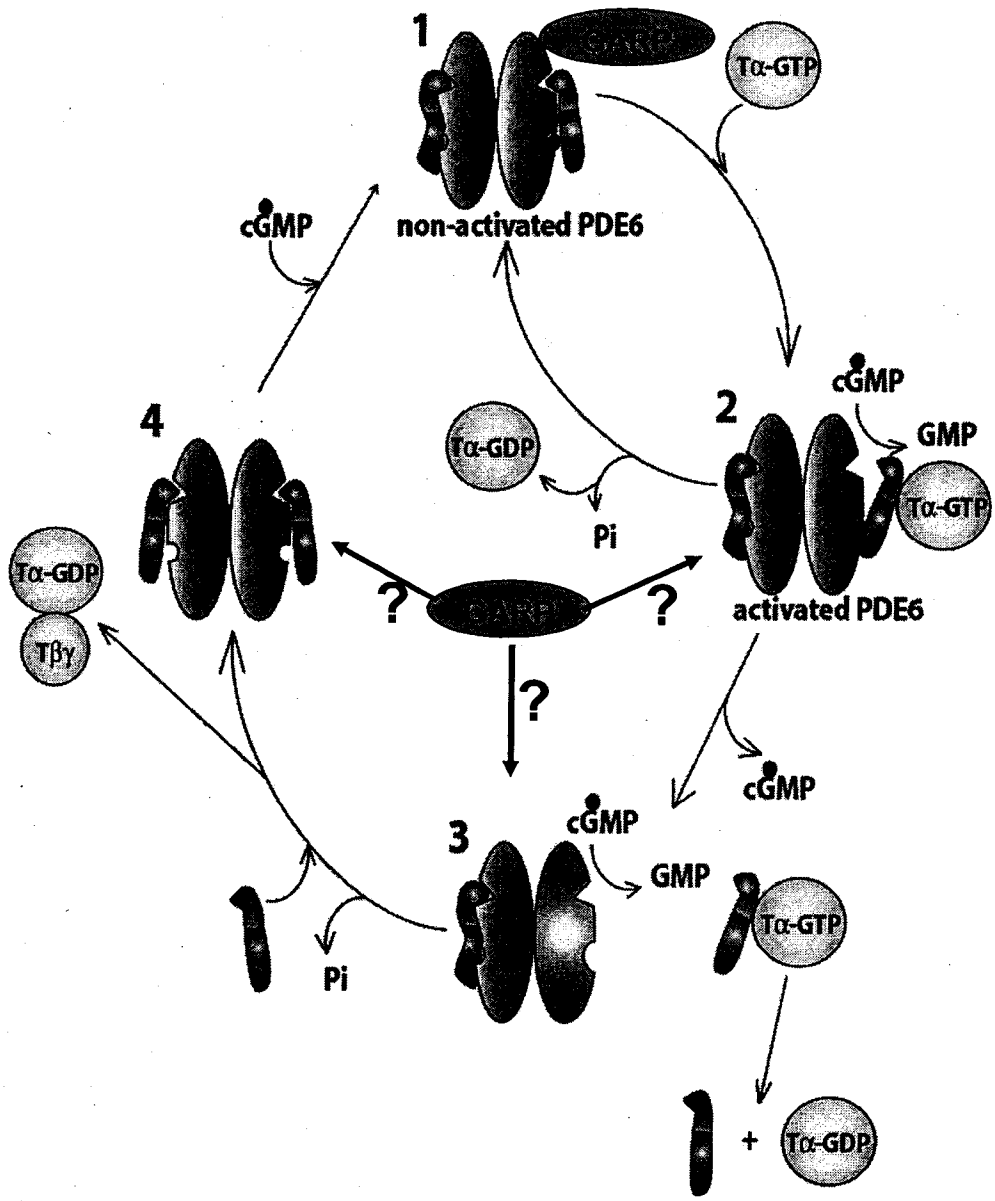

Figure 4.11. Potential role of GARP2 in PDE6 activation-deactivation cycle.
State 1. Dark-adapted state: PDE6 has two Pγ subunits bound, and the GAF domains are occupied by cGMP.
State 2. Initial transducin-activated state: Tα-GTP interacts with Pγ and releases inhibition at one catalytic site. cGMP still occupies the GAF domains.
State 3. Persistent transducin-activated state: as cGMP levels remain low, one GAF domain rapidly releases cGMP (the second GAF domain releasing bound cGMP more slowly). This enhances release of Tα-GTP-Pγ from PDE6. Subsequently, GTP is hydrolyzed to GDP and Pg dissociates from Tα-GDP.
State 4. Pγ re-associates with PDEαβ, but cGMP levels are still low and GAF domains remain empty until cGMP levels are restored to dark levels.

Upon activation by rhodopsin, transducin exchanges bound GDP for GTP, and the Tα subunit dissociates from its βγ subunits. Tα-GTP interacts with the C-terminus region of Pγ on the PDEαβγ$_2$ heterotetramer, and displaces the inhibitory constraint of Pγ on the catalytic site of PDE6. At the beginning of PDE6 activation, the cGMP level inside the cell is relatively high, and the GAF domains of the PDE6 holoenzyme are occupied. The occupancy of the GAF domains imposes a higher affinity of interaction for the N-terminus part of Pγ with PDEαβ, therefore, Pγ-Tα-GTP does not dissociate from the PDEαβ. This is depicted as State 2 in the activation cycle diagram (see Fig. 4.11.). To reproduce this activation state, the ROS membranes were first exposed to light, and the non-hydrolysable GTP analog, GTPγS was used. Extra cGMP was added to the ROS to ensure the occupancy of the GAF domains, and catalysis was inhibited with the PDE inhibitor, zaprinast. The membranes were then treated with 1% Triton X-100, and the soluble and insoluble parts were separated by centrifugation (see Methods). The protein content of the two fractions was analyzed by western blotting. PDE6 content was also determined by a PDE6 activity assay. Under this state of activation, ~50% of PDE6 appears to migrate to the detergent-insoluble portion of the membrane, while GARP2 remains soluble in 1% Triton X-100 (see Fig. 4.12.).

The next step in the activation cycle of PDE6 is achieved when the cGMP concentration drops inside the cell as a consequence of the catalytic activity of PDE6. When the cGMP level is low, the cGMP bound to the GAF domains is released. This release of cGMP from the GAF domains, causes Pγ-Tα-GTP complex to dissociate from the PDEαβ heterodimer (see Introduction). This state of activation is the State 3 in the diagram (see Fig. 4.11.). This state of the activation cycle can be reproduced by exposing

the cGMP depleted ROS membranes to light in the presence of GTPγS without cGMP added. Again, the resulting ROS membranes were treated with 1% Triton X-100, and the insoluble material was separated by centrifugation. Analyzing the GARP2 and PDE6 solubility, it is observed that the majority of PDE6, as well as GARP2 are soluble in this detergent condition (see Fig. 4.12.).

Upon Pγ-Tα-GTP release, the bound GTP is rapidly hydrolyzed to GDP by the intrinsic GTPase activity of Tα, which is enhanced by Pγ and another protein, RGS9. Only following GTP hydrolysis can Pγ dissociate from Tα and re-inhibit the catalytic site of PDE6 (see state 4 of Fig. 4.11.). To reproduce this last step in the PDE6 activation cycle, ROS membranes were first exposed to light in the presence of GTP. This permits PDE6 activation, but also inactivation can occur following GTP hydrolysis. Incubating activated ROS membranes for 2 hours at room temperature, cGMP levels become low and Pγ-Tα-GTP dissociates from PDEαβ. Upon this dissociation GTP is easily hydrolyzed to GDP, and Pγ is released. Once again, the membranes are treated with 1% Triton X-100, the insoluble parts separated by centrifugation, and proteins analyzed by western blotting or functional assay. PDE6 and GARP2 appear to be equally soluble by the detergent (see Fig. 4.12.).

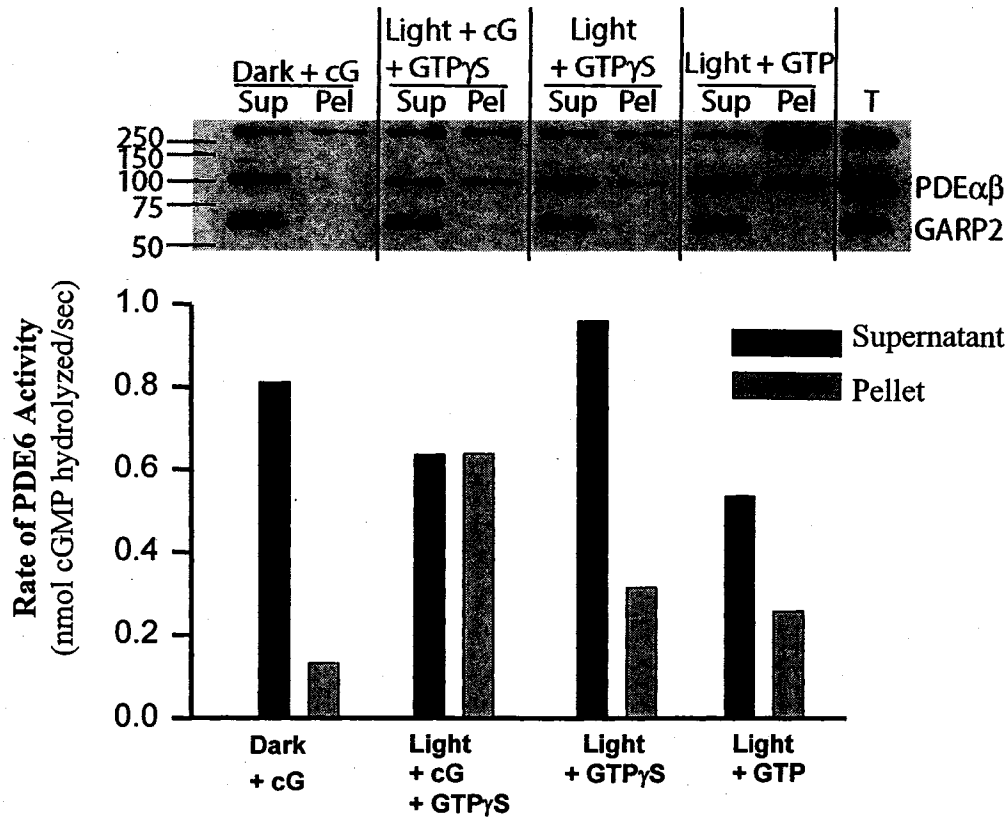

Figure 4.12. Enhanced membrane association of transducin-activated PDE6 is not accompanied by retention of GARP2 in a membrane-bound state.
ROS membranes (35 μM rhodopsin) were exposed to various treatments, and then mixed with 1% Triton X-100. The detergent-soluble and detergent-insoluble membrane fractions were separated by centrifugation. Pellets and supernatants were analyzed for immunoreactivity and PDE6 activity. (The band observed above 250 kDa marker represents the β-subunit of the cGMP-gated channel which immunoreacts with the GARP2 antibody.) This result is representative of two experiments.

Analysis of the membrane fractions under different states of PDE6 activation reveals that all the GARP2 and some of the PDE6 separate upon activation, particularly when the GAF domains are occupied by cGMP, and the efficiency of transducin activation is critical for phototransduction efficacy. In all conditions, some PDE6 and all GARP2 are soluble in the detergent. [Note that for the GTP only condition, the PDE6 activity analysis shows that there is more soluble PDE6 than membrane-bound, whereas

the western blot result shows different result that is probably an experimental error due to inaccurate sample loading on the gel.] These findings could suggest that GARP2 is only available to regulate PDE6 in its nonactivated state. It is important to mention that the separation of GARP2 from PDE6 immediately following activation would serve to maintain the high efficiency of phototransduction activation.

Summary

In this study we examined the mechanism of GARP2 interaction with PDE6. One site of interaction was determined to be with Pγ. Following GARP2 addition to the PDE6 holoenzyme, the affinity of Pγ for PDEαβ increased, lowering the probability of spontaneous activation of PDE6 holoenzyme. We determined that GARP2 interacts with two different regions of Pγ molecule, binding to both C- and N-terminal domains. GARP2 was also found to regulate the affinity of the GAF domains for cGMP, suggesting a direct interaction with the PDEαβ heterodimer. We also analyzed the possibility that GARP2 and PDE6 only interact at specific steps during light activation. Preliminary results showed that upon light activation, PDE6 and GARP2 separate on the surface of the disk membrane. Further studies are needed to determine all the light activation conditions that permit PDE6 and GARP2 to come in close proximity. It is also important to determine whether under the conditions that allow PDE6 and GARP2 to be in the same membrane fraction, they actually do interact in a physiologically meaningful way. One of the disadvantages of the detergent solubilization used in this chapter is that it disrupts the interaction of GARP2 with PDE6.

CONCLUSIONS AND FUTURE DIRECTIONS

The research presented here examined the regulation of rod photoreceptor PDE6 by the glutamic acid-rich protein 2 (GARP2). First we examined the proteins that interact with PDE6 during visual transduction. We developed methods to solubilize PDE6 from the disk membranes, and to isolate and identify the interacting protein complex. We developed methods to purify PDE6 free of some of the interacting proteins. Also we developed a method to prepare PDE$\alpha\beta$ heterodimer. We found that one of the proteins that interact with high affinity with PDE6 is GARP2. The experiments that will solidify the findings of the PDE6 interacting proteins include: the use of biotinylated antibodies for detection of association and dissociation of known proteins that interact with PDE6 during various stages of phototransduction, and identification of novel proteins by SDS-PAGE followed by mass spectrometry. For an unequivocal determination of PDE6 protein complex, all these experiments have to be pursued using the PDE6 solubilization methods described in Chapter 2.

After demonstrating that GARP2 is a high affinity PDE6 interacting protein, we studied the effect that GARP2 has on PDE6 activity. For this reason, we developed several methods for native GARP2 purification. We showed that native GARP2 is able to inhibit the PDE6 activity in its nonactivated state, but not when it is activated by either transducin or by limited trypsinization. This finding suggests that GARP2 has a role in reducing the spontaneous activation of rod PDE6, reducing therefore the "dark noise" of

rod photoreceptors, and confering a greater light sensitivity for rods compared to cones. Also, we demonstrated that GARP2 does not interfere with transducin's ability to activate PDE6, preserving the high efficiency of activation of visual excitation pathway.

We also found that GARP2 is able to alter the affinity of cGMP for the regulatory GAF domains of PDE6. It lowers the affinity of the high-affinity GAF domain, suggesting a direct interaction of GARP2 with PDE$\alpha\beta$ catalytic heterodimer, and also reverses the Pγ dependent high-affinity cGMP binding site to a low affinity site. We determined that GARP2 interacts with PDE6 through multiple sites, primarily at the level of Pγ subunit. We also showed that GARP2 increases Pγ affinity for the PDE$\alpha\beta$ dimer, determining the lower incidence of spontaneous activation of PDE6.

Because transducin activation of PDE6 is not affected by GARP2, we hypothesized that GARP2 and PDE6 separate during light activation. We reconstituted several stages of PDE6 activation with the use of nucleotides, and examined whether PDE6 and GARP2 were found in the same membrane fraction. Preliminary findings showed that PDE6 separates from GARP2 into detergent-insoluble membrane domains upon light activation. Further studies are needed to determine what stages of PDE6 activation-inactivation cycle permit PDE6 and GARP2 to interact. The presence of the interaction on native membranes will also have to be examined. The methods used for separating different membrane domains (i.e., lipid rafts)were found to disrupt PDE6-GARP2 interactions.

Some of the experiments that will have to be carried out to decipher the mechanism by which GARP2 interacts and regulates PDE6 during visual transduction cycle would include the following.

1. To define the regions of Pγ responsible for the GARP2 effect on PDE6 catalysis and regulation. This will first require the examination of GARP2 effect on PDEαβ heterodimer, followed by the analysis of GARP2 effect in the presence of N-and C-terminal Pγ peptides.

2. To determine the effect of GARP2 on the ability of transducin to activate PDE6 on GARP2-depleted ROS membranes.

3. To determine the affinity and stoichiometry of GARP2 binding to PDE6 catalytic subunits and Pγ using analytical ultracentrifugation.

4. To test the possibility of GARP2 being a transducin GTPase accelerating protein.

5. To test GARP2-PDE6 association at different steps of activation-inactivation cycle without the use of detergents. This will necessitate the use of immunoprecipitating antibodies.

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